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I am submitting herewith a thesis written by Matthew Richard Evans entitled "Phenotypic Incidence and Genotypic Diversity of *Listeria* Isolates Recovered from Dairy Cows, Calves, and the Farm Environment." I have examined the final paper copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Food Science and Technology.

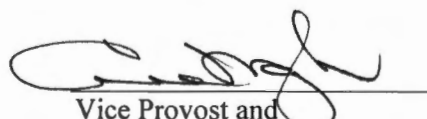

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**Phenotypic Incidence and Genotypic Diversity *Listeria* Isolates
Recovered from Dairy Cows, Calves, and the Farm Environment**

**A Thesis
Presented for the
Master of Science degree
The University of Tennessee, Knoxville**

Matthew Richard Evans
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Thesis

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DEDICATION

This thesis is dedicated to my parents

Mr. Thomas R. Evans

and

Mrs. Virginia H. Evans

who have given me invaluable educational opportunities.

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I would like to express my sincere appreciation to my advisor, Dr. F. Ann Draughon for her guidance, advice, support, and the opportunities she has provided me through the course of my graduate studies. I am extremely grateful to my committee members, Dr. P. Michael Davidson and Dr. Alan G. Mathew, for reviewing this manuscript. I would also like to thank them for their enduring assistance, useful suggestions, and for sharing their common sense advice with me. I thank the University of Tennessee Agricultural Experiment Station, The Food Safety Center of Excellence, and the United States Food and Drug Administration for supporting this research.

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ABSTRACT

This research was conducted as two separate studies. The objective of the first study was to determine the major sources of *Listeria monocytogenes* and *Listeria* spp. associated with dairy cows, calves, and the farm environment. Isolation and confirmation of *Listeria* spp. was performed according to the Food and Drug Administration's Bacteriological Analytical Manual (AOAC). Modifications in isolation protocol were made to determine the prevalence and optimal isolation media (MOX or PALCAM) for the detection of *L. monocytogenes* (LM) and other *Listeria* spp. from dairy farm cattle and environmental samples. Cows (n=30) (foremilk, hair, oral, rectal, and teats) and calves (n=30) (hair, oral, and rectal) were sampled over a six-month period. The environmental samples (n=20) included bulk tank milk, air, trough water (n=22), feed, bedding, farmyard soil, grain, silage, insects, milking equipment (cups, hoses, inflations), and river water (n=10) adjacent to the dairy farm. LM was more frequently isolated from the dairy farm environment (17.3%) than from dairy cows (13.1%) or calves (12.2%). All dairy cow, calf, and farm environmental sites, except for air, tested positive for the presence of LM. Based on the enrichment and isolation protocols evaluated in this research, the authors recommend that MOX be used for all sample types associated with dairy farms with the exception of soil, rectal, and trough water in which PALCAM gives better isolation of *L. monocytogenes*. The development of a stepwise "on-farm" pathogen reduction program to control LM in dairy cows and calves will be a challenge due to the frequent occurrence of LM in the environment of dairy cows. Protocols for sampling should be selected which can consistently detect the presence of LM.

In the second study, *Listeria* isolates (n=48) from a total of 548 cow, 454 calf, and 1,556 University of Tennessee Dairy Farm environmental isolates obtained during a 1999-2001 survey were submitted for automated ribotype analysis utilizing the Riboprinter microbial characterization system, alpha version (E. I. Du Pont de Nemours & Co., Inc.). This investigation

compared *Listeria* ribotypes (RTs) obtained from dairy cows, calves, and farm environmental isolates to a database of clinical isolates of *Listeria* associated with animal miscarriages and foodborne listeriosis. Riboprint patterns of 17 of the 48 isolates confirmed the identity as *Listeria* spp. based upon the Du Pont (*EcoRI*) database. Fourteen *Listeria* RTs were discriminated. The 14 RTs were identified as 5 *L. monocytogenes* RTs (1029, 1041, 1051, 1052, and 1XXXX, a confidential isolate held by Du Pont), 5 *L. innocua* RTs (1005, 1006, 1009, 1010, and 1019), and 4 *L. welshimeri* RTs (1072, 1073, 1074, and 1079). Of the isolates selected for ribotyping, *L. monocytogenes* was detected phenotypically from grain, feedbunk, corn silage, raw bulk tank milk, air, soil, bedding, river water adjacent to the midpoint of the farm, milking equipment, calf oral and hair samples, as well as cow anal, oral, and teat samples. According to ribotype analysis, optimal detection sites for the presence of *L. monocytogenes* were from the cows' teats, the oral cavity of calves, corn silage, raw bulk tank milk, and from the river water adjacent to the midpoint of the farm. Knowledge of the agricultural ecosystem, the taxonomy of *Listeria* strains, and careful attention to detailed isolation and confirmation protocols were essential to prevent misidentification of *Listeria* spp. when initiating and conducting an on-farm pathogen-reduction program.

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PART I
LITERATURE REVIEW

INTRODUCTION

Listeria is recognized as the causative agent of listeriosis. Listeriosis is a zoonotic illness affecting humans and animals. *Listeria* was documented in the scientific literature as early as 1891 when small, Gram-positive, rod-shaped organisms in human tissue were observed by Hayem (Donnelly, 1994). Characterization of the suspect organism, now known as *Listeria monocytogenes*, was achieved by Murray et al. (1926) noting monocytosis and hepatic lesions in rabbits implicated in an epizootic outbreak in 1924. Subsequent characterization of this organism by Murray identified a novel species, which he called *Bacterium monocytogenes* (McLaughlin, 1987; Gellin and Broome, 1989; Low and Donachie, 1997).

Human listeriosis was first reported by Nyfeldt in 1929 when he isolated a gram-positive bacillus from a case of infectious mononucleosis, which he called *Bacterium monocytogenes hominis* (Gellin and Broome, 1989). Although, the monocytosis that Nyfeldt observed was later found not to be common to human listeriosis. Pirie observed unusual deaths in gerbils near Johannesburg, South Africa in 1927 and isolated a Gram-positive bacillus, which he called Tiger River disease (Ryser and Marth, 1999). He later named the agent *Listerella hepatolytica* after Lord Lister, a distinguished bacteriologist. In 1939, a Commission of the Committee on Systematic Bacteriology rejected the name *Listerella* because it was previously given to two other groups of organisms (Ryser and Marth, 1999). Pirie renamed the bacterium as *Listeria monocytogenes* in 1940 (McLaughlin, 1987; Donnelly, 1994). *Listeria monocytogenes* has been a major concern for the food industry for more than two decades (Rocourt and Cossart, 1997). Frequent recalls of prepared foods due to contamination with *L. monocytogenes* have caused multi-million dollar losses for many food companies (Ryser and Marth, 1999).

GENERAL CHARACTERISTICS

The genus *Listeria* currently includes six species: *L. monocytogenes*, *L. ivanovii*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, and *L. grayi* (Ryser and Marth, 1999). *Listeria* is a small

(0.5 µm in diameter and 1-2 µm in length), Gram-positive, nonsporeforming, coccoid rod, which can occur singly and in V-shaped and Y arrangements. In older (>48 hours) or osmotically shocked cultures, cells appear long, thin, and filamentous. The bacterium is aerobic to facultatively anaerobic, however the organism will typically grow profusely in an anaerobic to microaerophilic (5% O₂, 10% CO₂, and 85% N₂) environment (Ryser and Marth, 1999; Donnelly, 1994).

Four peritrichous flagella allow *Listeria* to be motile when cultured at 20-25°C. Flagellin is produced and assembled at the cell surface, which allows the microorganism to display characteristic tumbling and umbrella motility. At 37°C, flagellin production is significantly reduced, hence *Listeria* is weakly motile or not motile at all (Farber and Peterkin, 1991; Ryser and Marth, 1999). When *Listeria* is stabbed into semisolid motility media and incubated at 20-25°C for 48 hours, classic inverted “pine-tree” or “umbrella” growth can be observed 0.5 cm below the surface of the medium (Ryser and Marth, 1999). *Listeria* grow best within the pH range of 6-8, however *L. monocytogenes* species strains will generally grow in substrates over a range of 4.1-9.6 (Jay, 2000). *Listeria monocytogenes*, a psychrotrophic pathogen, will grow over the temperature range of 1-45°C, with optimal growth occurring in the range of 30-37°C (Jay, 2000). Shahamat et al. (1980) reported that *L. monocytogenes* could survive at 37°C for 15 days in 10.5% NaCl and in tryptic soy broth (TSB) containing 25.5% NaCl for 32 days at 4°C.

The majority of *L. monocytogenes* strains are catalase-positive, although some strains have been observed to be catalase negative (Hagen et al., 1998). *Listeria monocytogenes* is oxidase-negative and hydrolyses esculin as well as sodium hippurate (Ryser and Marth, 1999). Additionally, all species and strains are methyl red and Voges-Proskauer test positive (McLaughlin, 1987; Ryser and Marth, 1999). *L. monocytogenes* is known to produce acid via fermentation of fructose, rhamnose, mannose, salicin, maltose, dextrin, and alpha-methyl-D-glucoside. Acid production is not generally observed from mannitol, xylose, adonitol, arabinose,

dulcitol, erythritol, glycogen, inositol, inulin, melibiose, raffinose, or sorbose. A variable acid fermentation reaction is produced from galactose, lactose, melezitose, sorbitol, starch, sucrose, and trehalose (Ryser and Marth, 1999).

On 5% sheep or horse blood agars, *L. monocytogenes* exhibits beta-hemolysis, which may be weak. However, hemolytic activity is enhanced when inocula are stabbed into the blood agar instead of being streaked onto the agar surface. The CAMP (Christie, Atkins, and Munch-Petersen) test is considered by many researchers to be the definitive test for *L. monocytogenes*. A *Listeria* isolate must be considered a presumptive *L. monocytogenes* isolate if it is CAMP positive in the presence of *Staphylococcus aureus* or *Rhodococcus equi*, however, the presumptive isolate of *L. monocytogenes* is not necessarily a virulent one (McKellar, 1994). Hemolytic stimulation on sheep or horse blood agar plates in the presence of *S. aureus* may be due to the interaction of a phosphatidylinositol-specific or phosphatidylcholine-specific phospholipase C from *L. monocytogenes* and a sphingomyelinase from *S. aureus* (McKellar, 1994). A brief summary of differential characteristics of the species within the genus *Listeria* is illustrated in Table 1.

LISTERIOSIS IN HUMANS

Unlike infection with other foodborne pathogens, listeriosis is often severe and is associated with a mortality rate ranging between 20-50% (Ryser and Marth, 1999; Fleming et al., 1985; Low and Donachie, 1997). Individuals at greatest risk for contracting listeriosis include pregnant women, neonates, and immunocompromised persons (including those with HIV organ transplant patients, cancer, the elderly, and those on corticosteroid therapy) but may also occur in persons with no immunosuppressive disorder (Schuchat, et al., 1992). Increased risk of contracting listeriosis has also been found among individuals who used antacids, H₂-blocking agents or laxatives in the month prior to their exposure to *L. monocytogenes*, thereby altering the environment of the gastrointestinal tract (Schuchat et al., 1992).

Table 1. Typical differential characteristics of the species of the genus *Listeria*^{a,b,c}

Characteristics	<i>L. grayi</i>	<i>L. innocua</i>	<i>L. ivanovii</i>	<i>L. monocytogenes</i>	<i>L. seeligeri</i>	<i>L. welshimeri</i>
β-Hemolysis	-	-	+	+	+	-
CAMP test (<i>Staphylococcus aureus</i>)	-	-	-	+	+	-
CAMP test (<i>Rhodococcus equi</i>)	-	-	+	-	-	-
Acid production from:						
Mannitol	+	-	-	-	-	-
α-Methyl-D-Mannoside	ND ^d	+	-	+	-	+
L-Rhamnose	-	+/- ^e	-	+	-	+/-
Soluble starch	+	-	-	-	ND	ND
D-Xylose	-	-	+	-	+	+
Hippurate hydrolysis	-	+	+	+	ND	ND
Reduction of nitrate	-	-	-	-	ND	ND

^a Symbols: +, 90% or more of strains are positive; -, 90% or more of strains are negative; (+/-), 11- 89% of strains are positive (1994).

^b The species *Listeria denitrificans* has been transferred to a separate genus *Jonesia* in Group 20 in the Bergey's Manual of Systematic Bacteriology.

^c ND, not determined

^d All species within the genus *Listeria* are Gram-positive rods, are positive for the production of catalase, and motile (usually characteristic "umbrella motility") at 22-25°C, however motility does not typically occur at 37°C.

^e Partial fermentation of the sugar

^f Usually a wide zone or multiple zones

^g A few strains negative

As adopted and modified from (Holt et al., 1994).

Listeriosis has an extended incubation time (up to 5 weeks) and manifests itself with a broad range of symptoms (Rocourt and Cossart, 1997). Infection in a pregnant female can be asymptomatic or characterized by an illness with varying symptoms such as, fever, myalgia, muscular pain, and/or headaches. The fetus or infant may suffer more serious consequences such as, spontaneous abortion, fetal death, still-birth, severe neonatal septicemia and meningitis. In adults, meningitis, meningioencephalitis, and septicemia are frequent in cases of listeriosis, although other organ infections and gastroenteritis could also occur (Rocourt and Cossart, 1997). *L. monocytogenes* infections of the skin have arisen without systemic involvement in farmers or veterinarians who have delivered infected or aborted calves or in butchers exposed to contaminated meat products (Molsan et al., 1998).

The most significant virulence factor associated with *L. monocytogenes* is listeriolysin O (LLO). This substance is responsible for beta-hemolysis of erythrocytes and the destruction of phagocytic cells. Because *L. monocytogenes* is an intracellular pathogen it must be able to enter susceptible cells and replicate within these cells. LLO aids in this process. Once inside a host cell, *L. monocytogenes* is able to invade adjacent host cells by forming filopodiums or projections, which are absorbed by adjacent cells and the invasion process is repeated. In this way, *L. monocytogenes* can survive intracellularly and spread from cell to cell without having to leave a host cell (Jay, 2000).

FOODBORNE LISTERIOSIS

The first documented outbreak of foodborne listeriosis in North America occurred in eight hospitals near Boston, Massachusetts in 1979 among patients who had consumed lettuce, carrots, and raw celery (Ho et al., 1986). The exact source of infection in this outbreak is unknown since no attempts were made to isolate *L. monocytogenes* from the vegetables involved (Ryser and Marth, 1999). In the Maritime Provinces of Canada an outbreak of listeriosis occurred after contaminated cabbage was marketed and then consumed by the public in the form

of coleslaw (Schlech et al., 1983). The investigation traced the product back to the farm where sheep manure was used to fertilize the cabbage fields. Shortly before this incident, two sheep from the same farm had died of listeriosis. In 1983, a specific brand of pasteurized milk was linked to an outbreak of listeriosis in Massachusetts. The milk came from a cluster of farms where there had been an outbreak of listeriosis in dairy cattle. At the time of the milkborne outbreak, *L. monocytogenes* was isolated from the raw milk on these farms and there was evidence of improper pasteurization procedures at the milk processing plant (Fleming et al., 1985). This investigation was the first to suggest the potential for greater heat resistance of *L. monocytogenes* (Fleming et al., 1985), which was later debated (Donnelly and Briggs, 1986). In 1994, *L. monocytogenes* was implicated in an outbreak of listeriosis that occurred in Illinois after the consumption of chocolate milk. The source of the pathogen was traced to the improper sanitation of mechanical equipment within the processing plant and poor post-processing refrigeration practices (Dalton et al., 1997).

The addition of raw milk in the processing of Hispanic cheese at a factory in Southern California in 1985, led to a massive outbreak of human listeriosis and a 34% death rate among those consumers stricken with this illness (Linnan et al., 1988; Farber and Peterkin, 1991). In response to the 1985 Mexican cheese incident in California as well as a smaller outbreak in Switzerland, Swiss officials initiated a survey to determine the incidence of *Listeria* in various cheeses and dairy products. This study revealed the presence of *L. monocytogenes* in Vacherin Mont d'Or cheese. Upon further investigation, Swiss officials traced the source of the outbreak to an infected dairy herd seven years prior to the initial case of listeriosis in 1983 (Bille, 1990; Farber and Peterkin, 1991). In 1995 and 1997, consumption of Brie de Meaux Cheese, which is a raw milk soft cheese, was implicated in an outbreak of listeriosis that occurred in France and Normandy, respectively. In both cases, *L. monocytogenes*-contaminated raw milk was used to produce the soft cheese (Ryser and Marth, 1999).

There have been numerous other outbreaks of listeriosis related to mishandling of foodstuffs at the manufacturing and retail levels involving the following products: pate' (1987-89), soft cheese (1987), goat's milk cheese (1988), cooked chilled chicken (1988) in the United Kingdom; pork tongue in jelly (1992) and pork rilletes (1993) in France; turkey franks (1988), shrimp (1989), and wieners and/or deli meats (1998-99) in the United States (Jay, 2000). Since the incubation period for listeriosis may be as long as five weeks, it is often difficult to determine the contaminated food source. In addition, by the time an individual is stricken with the disease, the suspected food product may no longer be available for microbiological testing (Gellin and Broome, 1989; Pinner et al., 1992). High mortality rates among humans, financial losses due to product recalls (Ryser and Marth, 1999), and lawsuits suffered by the dairy and meat industries have necessitated the promulgation of a "zero-tolerance" policy for the presence of *Listeria monocytogenes* in and on all cooked/ready-to-eat (RTE) foods and dairy products by the United States government. The Food and Drug Administration (FDA) and the United States Department of Agriculture (USDA) define "zero-tolerance" as the absence of *Listeria monocytogenes* in 50g samples. However, "zero-tolerance" generally means the absence of *Listeria monocytogenes* in 25g samples (Jay, 2000). Sporadic or isolated cases of listeriosis may go unreported to the Centers for Disease Control (CDC), Atlanta, GA, thus reducing early detection and monitoring of *L. monocytogenes* within the food industry.

SOURCES OF *LISTERIA*

Dairy Cattle

The incidence of listeriosis in ruminants has significantly increased over the last few decades (Low et al., 1992). The spread of listeriosis to dairy cattle usually occurs through transmission by feed, with silage frequently implicated as the vector (Vazquez-Boland et al., 1992; Ryser and Marth, 1999; Wiedmann et al., 1997a; Fensterbank et al., 1984). The infectious process in dairy cattle usually occurs in the viscera, brain or the gravid uterus pathway. Since *L.*

monocytogenes is present in soil, fecal material, vegetation, and cattle bedding, alternative routes of infection may include abrasions of the nostrils or the conjunctiva while grazing in pastures, eating baled silage, or via the teat of a lactating cow (Ryser and Marth, 1999; Garcia et al., 1996). As a result of silage particles coming into contact with faces of cattle during feeding, direct inoculations of *L. monocytogenes* have been known to enter the conjunctiva of the eye, which result in infections lasting up to three weeks (Morgan, 1977). *Listeria monocytogenes* may also be shed in milk without any abnormal appearance of the milk or symptoms of mastitis (Ryser and Marth, 1999). To avoid spreading of infections, separating infected cows with clinical mastitis that do not respond to treatment is considered a prudent measure (Low and Donachie, 1997).

The first description of neurological disease due to *L. monocytogenes* occurred among sheep in New Zealand in 1929, which local dairy farmers called ‘circling disease’ after the type of staggering gait demonstrated by the stricken animals (Low and Donachie, 1997). The incubation period may be as long as six weeks for listeriosis (Ryser and Marth, 1999). When *L. monocytogenes* attacks the central nervous system, paralysis of the mouth, throat, and face may occur. Other symptoms may include: drooping of the lips, ears, and eyelids depending upon which cranial nerves are affected. As vision and mobility become impaired, the animal takes on a circling type of locomotion. Finally, the animal lapses into a coma and may die within two days. During this period, there are varying degrees of fever. Most cattle survive 4-14 days after the onset of early symptoms (Ryser and Marth, 1999).

Infection with *L. monocytogenes* is frequently associated with abortion in cattle and usually occurs in the last trimester of pregnancy (Low and Donachie, 1997). *Listeria /monocytogenes* may be transmitted to the calf during the birthing process causing septicemia or meningitis and death shortly after birth. Generally, the organism is transferred to the fetus through the placenta resulting in abortion or stillbirth (Low and Donachie, 1997).

CONTAMINATION SOURCES

River water

Water run-off from the agricultural ecosystem, as well as the increasing use of land-bound waterways for recreation, transportation, industrial and sewage treatment effluents, may result in the increasing presence of *L. monocytogenes* in lakes, rivers, and streams. In May 1993, 19 samples (100 ml) from various sites of the River Don in Scotland revealed that 42% of these samples were positive for *L. monocytogenes*. All of the positive samples were retrieved from the upper reaches of the river and numbers of *L. monocytogenes* ranged from 10 to 350 CFU/L. Six months later in December, sampling 17 of the sites showed that 53% of the samples were positive for *L. monocytogenes* and the positive samples were evenly distributed over the length of the river. The counts of *L. monocytogenes* ranged between 10 and 80 CFU/L (Fenlon et al., 1996).

In New South Wales (Australia), 61% of 27 samples collected from the course of the Windsor (41%) and Georges Rivers (20%) tested positive for *Listeria* spp. Of the 27 samples 33.3% were confirmed as *L. monocytogenes*. The authors surmise that the higher incidence of positive samples from the Windsor River was due run-off from farmlands located along its banks (Arumugaswamy and Gibson, 1999).

Silage

Silage is prepared from grass, maize, whole crop cereals, or leguminous plants, which are dried in the field usually to 60-70% moisture content, mechanically chopped, and ensiled (Ryser and Marth, 1999). Anaerobic conditions catalyze the indigenous or inoculated lactic acid bacteria to rapidly multiply to levels of about 10^9 CFU/g within 48 hours (Ryser and Marth, 1999). The lactic acid bacteria convert plant sugars to lactic acid resulting in a quick decrease in pH. Generally, well-preserved silage has a $\text{pH} \leq 4.5$ (Ryser and Marth, 1999). The acidic environment of silage inhibits the growth of both spoilage microorganisms as well as *Listeria* as long as an anaerobic condition is sustained. Low moisture silages have a higher pH and less available water

compensating for the preservative effect of low acid silage (Ryser and Marth, 1999). Silages in a cool, wet climate have low sugar levels and a high moisture content resulting in a slower fermentation. These conditions often lead to poor-quality silage, which is susceptible to *Listeria* contamination, especially if strict anaerobic conditions are not maintained during the ensiling process (Ryser and Marth, 1999).

It has been well documented that listeriosis in ruminants is associated with the feeding of poor-quality silage (Gray, 1960a; Fenlon, 1986b; Grant et al., 1995). In 1922, Palsson, an Icelandic scientist, warned against a potent disease, now known as listeriosis, that occurred in ruminants after silage feeding. The coincidental feeding of silage and the onset of illness among cattle in Iceland prompted farmers to call this disease “votheysveiki” or silage sickness (Gray, 1960a; Donnelly, 1994). Jones and Little diagnosed the first case of listeriosis infection in cattle in the United States in 1934 (Amstutz, 1980). Olafson, in 1940, observed a relationship between silage consumption and “*Listerella*” encephalitis in ruminants (Ryser and Marth, 1999).

Gray (1960a) investigated two instances of epizootic bovine abortions in eastern Montana in the spring of 1957 and 1958, which followed the feeding of corn silage. He isolated *L. monocytogenes* from a non-pregnant mouse and from the fetus of a pregnant mouse, both of which he fed the corn silage. Cultures from the bovine fetuses and mice were serologically identical for *L. monocytogenes* type 1 (Gray, 1960a). Poor quality silage (pH>4.5) was therefore implicated in the death and abortion of cattle on the ranch in Montana and strengthened the association between feeding of silage and listeriosis.

Work done by Gronstol (1979) established a relationship between the pH and the presence of *L. monocytogenes* in grass silage. Grass silage samples (n=291) were collected from tower silos, pit, and clamp silages on 113 farms with recent outbreaks of listeriosis in ruminants (Gronstol, 1979). Clamp silage is packed into a long cylindrical shape on top of a concrete slab and covered with a plastic sheet, which is tightly sealed at both ends by clamps (Ryser and Marth,

1999). *Listeria monocytogenes* was isolated from 22% of the samples with a pH <4, from 37% with a pH 4 to 5, and from 56% with a pH >5 (Gronstol, 1979). A similar study was carried out on a farm with no outbreaks of listeriosis and revealed that *L. monocytogenes* was isolated from 28% of 32 grass silage samples (Gronstol, 1979).

An increase in the popularity of big bale silage from the mid-1970's to the mid 1980's in the UK corresponded to a rise in the cases of listeriosis infections in sheep from 44 (1975) to 269 (1984) (Fenlon et al., 1989). Silage in plastic bags, as well as wrapped bales, otherwise known as "big bale silage," is more liable to contamination by *Listeria*. Levels of acidity are not as high as conventional clamp silage because it is difficult to get an airtight seal in big bale bags. Thus, fermentation is incomplete because of the resulting lower density of big bale silage (Fenlon et al., 1989). A two-year (1983-1984) examination of silage in Scotland, showed that the incidence of *L. monocytogenes* and *L. innocua* in clamp silages was 2.5% and 10.9%, respectively. The occurrence of *L. monocytogenes* and *L. innocua* in big bale silages was 5.9% and 17.8%, respectively. When moldy silage was selected from clamped or baled silage, the prevalence of *L. monocytogenes* reached 44% (Fenlon, 1985).

A year later, Fenlon (1986b) analyzed the surface layer and edges (pH 8.3-8.5) of clamp silage, implicated in a small outbreak in calves, for the presence of *Listeria* species. The first 1-2 cm of the clamp silage contained *L. monocytogenes* in excess of 12,000 CFU/g while 15 cm in from the surface of the heap of silage where the pH was 4.5, *Listeria* spp. were not recovered. Using six 500-g laboratory grass silage bales wrapped in plastic bags, Fenlon (1986a) found that 75 mm in from the tie end (end of the bag which has a tied closure) to the center of the bale and towards the rear of the bale, the pH remained at 3.8. The silage was of good visual quality (no mold growth) and was found to be free of *Listeria* species. Around the tie end of the bag, where air could easily infiltrate, the silage was moldy and *L. monocytogenes* was detected at levels of $>1.1 \times 10^6$ CFU/g after 3 to 6 weeks.

Fenlon (1986a) listed several important factors to be considered when creating an effective silage fermentation that inhibits the growth of *Listeria*. The microorganism apparently occupies a microaerophilic niche between the stable anaerobic silage and aerobic decomposition. In better quality silages, lactic acid, causes a rapid decrease in pH producing a good-quality, well-preserved silage with a $\text{pH} \leq 4.5$. These acidic conditions retard spoilage and *Listeria* as long as anaerobic conditions remain constant (Fenlon, 1986a; Fenlon et al., 1986b). A few inches from the surface of a silage bale or clamp the aerobic environment provides good conditions for growth of *L. monocytogenes*. Deeper into the silage, anaerobic conditions and fermentation of the natural sugars, create and maintain an acidic environment, which is an unfavorable habitat for *L. monocytogenes* (Fenlon, 1985). *L. innocua* and *L. monocytogenes* were isolated from 15.3% and 2.9%, respectively, of the silage samples taken from approximately 10% of Vermont dairy farms (Perry and Donnelly, 1990). The authors concluded that as silage pH increased the proliferation of *Listeria* increased, correspondingly. When the pH was <4.0 , 7.9% of silage samples were positive for *Listeria* spp., but if the pH rose to between 4.0 and 4.99, 20.3% of samples contained *Listeria* spp. At pH 5.0 to 5.99, 52.9% of the samples were positive and, at $\text{pH} > 6.0$, *Listeria* spp. were present in 100% of samples. Skovgaard and Morgen (1988) surveyed seven dairy farms in Jutland, Denmark for *L. monocytogenes* in feeds. *Listeria monocytogenes* was found in four types of feedstuff: $\text{pH} > 4.5$ silage, $\text{pH} \leq 4.5$ silage, NH_3 -treated (alkaline treated) straw, and other dairy cattle feed (hay, mash, and beet debris) with an incidence of 63%, 67%, 63%, and 50%, respectively. According to Ryser and Marth (1999), dry matter silages tend to have a higher pH, but the reduced amount of water compensates for the preservative effects caused by reduced acidity.

The effects of a balance between the physico-chemical and microbiological characteristics (i.e.: oxygen tension, pH, dry matter), which influence the growth and survival of *L. monocytogenes* in silage was demonstrated by Donald et al. (1995), who infused laboratory

ensiled grass with gas mixtures containing 0-5% oxygen concentrations. Prior to ensiling, the chopped grass samples were inoculated with *L. monocytogenes* to give a final concentration of 10^3 to 10^6 CFU/g grass. Of the grasses ensiled, an oxygen concentration $\geq 1.0\%$ sustained growth of *L. monocytogenes*. Below 1% oxygen, growth depended on the quality of fermentation. At 0.5% oxygen, the survival of *L. monocytogenes* was prolonged while 0-0.1% caused *L. monocytogenes* to die off. Ensiled grass of poor quality (pH>4.5) (grass cut down late in the season) with a restricted fermentation prolonged the growth of *L. monocytogenes* even under anaerobic conditions (Donald et al., 1995).

The low moisture content in the majority of animal feeds, such as hay and cereal grains, reduces the growth of *L. monocytogenes*. Although *L. monocytogenes* has been found in grain and hay, it has not been found in quantities that would likely create a serious health threat to animals. Ewe's feeds collected every three months for one year yielded contamination rates of 1.2%, 11.9%, and 1.2% by *L. grayi*, *L. innocua*, and *L. monocytogenes*, respectively (Garcia et al., 1996). *L. monocytogenes* was present in all dried commercial feed grain samples, 59% of the hay samples, and was absent from silage samples. *Listeria* spp. were detected in 62.5% of hay samples, 12.5% of silage samples, and below 100 CFU/g in feeds (Garcia et al., 1996).

Wild animals

There is long-term and continuous scientific documentation to suggest a strong cause-and-effect relationship between silage and animal listeriosis. Research suggests that many healthy wild and domestic animals may be carriers of *L. monocytogenes* and may shed it in normal body discharges (Gray, 1960b). Hence, any of the small wild animals (i.e. rodents, birds) could contaminate feeding areas (i.e., silage and grain pits, feedbunks, and watering troughs) as they scavenge for food and water (Amstutz, 1980). The prevalence of *Listeria* in feeds has been suggested to be associated with high numbers of *Listeria* being isolated from the feces of dairy cows (Skovgaard and Morgen, 1988). Although the numbers of *Listeria* present in feed samples

taken from four farms in Finland were below 100 CFU/g of feed, *L. monocytogenes* and *L. innocua* were isolated from feed troughs and feed dispensing machines on all four farms (Husu et al., 1990). The author attributes the low numbers of *Listeria* present in the fecal samples from dairy cows to the low numbers of *Listeria* present in the feed combined with an effective immune system of the animals (Husu et al., 1990).

Watering troughs

There has been little information published regarding isolation of *Listeria* from the semi-stagnant, microenvironment of cow watering troughs that exist within the confines of the farm environment. The watering trough may be visited numerous times a day by dairy cattle and calves that are often identified as both reservoirs and carriers of *Listeria*. Garcia et al. (1996) reported that, of 51 ewe drinking water trough samples, 31.7% tested positive for *L. innocua* and 7.8% tested positive for *L. monocytogenes*. Furthermore, one sample contained both *L. grayi* and *L. innocua*, and in a second sample both *L. innocua* and *L. monocytogenes* were present. Husu and colleagues (1990) have reported a higher incidence of *L. monocytogenes* (48.0%) and of *Listeria* spp. (52.0%) in dairy cow watering cups.

Raw milk

Previous research in various countries has found *L. monocytogenes* in raw cows' milk with a distribution ranging from approximately 0.1 to 45% (Ryser and Marth, 1999). *L. monocytogenes* can be shed in the milk of apparently healthy dairy cows (Husu, 1990; Skovgaard and Morgen, 1988) and thus may pose a threat to public health (Ryser and Marth, 1999).

As early as 1938, Schmidt and Nyfeldt proposed that drinking milk from mastitic cows may have caused a small outbreak of human listeriosis in Denmark (Ryser and Marth, 1999). Working in Sweden in 1944, Wramby isolated *L. monocytogenes* from the milk and udders of mastitic cows (Ryser and Marth, 1999). Jensen and colleagues (1996) examined quarter milk samples from 1,132,958 cows from 36,199 herds over the course of a 23-year period (1972-1994)

for the presence of *Listeria monocytogenes*. The percentage of cows infected with *L. monocytogenes* ranged from 0.01 to 0.1% and herds with an infected cow ranged from 0.2 to 4.2%. This shows a low, but constant level of infection (Jensen et al., 1996). Weekly analysis (11/96-11/97) of the right hindquarter of a 7-year old, silage fed, Simmental cow revealed the repeated excretion of *L. monocytogenes* (Wagner et al., 2000). Listerial cell counts from the right hindquarter of the cow demonstrated 40,000 CFU/ml of milk during peak lactation and 80,000 CFU/ml from the foremilk alone. Silage monitoring initiated in May 1997, demonstrated contamination of the silage with *L. monocytogenes* at 100 CFU/g (Wagner et al., 2000). The feeding of silage contaminated with *L. monocytogenes* can result in the asymptomatic shedding of this organism in the milk of dairy cows (Donnelly, 1986).

A study by Fedio and Jackson (1992) monitoring 401 cow milk samples via direct milking of each quarter (not including the foremilk) showed that 5.2% of these samples were positive for *L. monocytogenes*. However, only one quarter of one cow was shown to have sub-clinical listerial mastitis of the 262 aseptic quarter samples collected. Although the organism was virtually absent from the interior of the udder, 5% of the cow milk samples still tested positive for *L. monocytogenes*. The authors concluded that *L. monocytogenes* was gaining access to the cow milk samples from the outer surfaces of the teats from the farm environment, or via unsanitary milking equipment. Therefore, animals with listerial mastitis may disseminate the organism to other animals on the farm during the milking procedure (Fedio and Jackson, 1992).

During an outbreak of listeriosis in Massachusetts and Connecticut in the summer of 1983, which was linked to pasteurized whole 2% milk, scientists from the CDC, analyzed 121 raw milk samples and 14 milk socks (filters) and detected *L. monocytogenes* in 12% of the milk specimens and 14% of the milk socks (Hayes et al., 1986). Wnoronski (1990) isolated *Listeria* spp. from 7.6% (5.2% were *L. monocytogenes*) of 961 bulk tank milk samples and from 85.7% (81.0% were *L. monocytogenes*) of 21 raw milk dairy factory silos. Other studies have found *L.*

monocytogenes to be present in 2.73% of 1,720 random farm bulk tanks in Ontario, Canada (Steele et al., 1997) and 45.3% of the raw bulk tank samples taken during a one-year period from a single dairy in Spain (Fernandez-Garayzabal et al., 1987). The highest isolation rates, in the latter study were shown in December (75%) and February (75%) and the lowest in June (20%) and August (14.3%) (Fernandez-Garayzabal et al., 1987).

Rea and associates (1992) examined raw milk from 70 farms in Ireland over a 13-month period. Of the 589 total samples collected, 8.3% were positive for *Listeria* spp., 4.9% of the samples were positive for *L. monocytogenes* and 3.4% for *L. innocua*. The researchers noted that the incidence of *L. monocytogenes* was higher in the winter when cows were housed indoors than in the summer (Rea et al, 1992). A six-month survey of raw bulk milk from farms in Camembert, Normandy, showed that of 69 bulk milk samples collected, 36% were positive for *Listeria* species. Of these positive samples, 76% were collected in the winter when cows were kept in sheds and *L. monocytogenes* was detected in 6% of these winter isolates (Desmaures et al., 1997). In the United States, 292 bulk tank milk samples from farms in East Tennessee were tested and *L. monocytogenes* was isolated from 4.1% of the samples. Raw bulk tank milk was consumed by 68 of 195 (35%) of dairy producers in this study (Rohrbach et al., 1992).

Ewe's milk samples were analyzed for the presence of *Listeria* over a one-year period in Madrid, Spain. Samples were taken from 287 farm bulk tanks and 17 transport tankers where *L. monocytogenes* and *L. innocua* were detected in 2.19% and 2.00% of 1052 farm samples, and in 18.38% and 11.76% of 136 tanker samples, respectively. Detection of *L. grayi*, *L. ivanovii*, *L. seeligeri*, and *L. welshimeri* was below 0.4% in bulk tank samples and under 1% in tanker samples. Interestingly, the authors state that ewes' milk contamination by *L. monocytogenes* and other *Listeria* spp. was markedly increased on farms where cows were raised as compared to farms where only ewes were present (Rodriguez et al., 1994).

Husu et al. (1990) observed that it was not unusual for the teats of cows to be partially covered with fecal material, bedding, and soil, hence *Listeria* was often isolated from skin of the teats and before milking. Swab samples taken from the teats of 59 cows, both before and after washing and drying the udder. No *Listeria* were detected from the teat swabs taken after washing and drying of cow udders. *Listeria* species were isolated from 30.4% of the samples taken before cleaning the teats on one of the four farms in this study, which had the largest barn. However, *L. monocytogenes* was detected from 11.1% of the samples taken before cleaning the teats, on a farm, which had the smallest barn. Therefore, proper washing and drying of the udder before milking is essential to the elimination of *Listeria* (Husu et al., 1990). Contamination of bulk milk may occur in one of two ways; directly as a result of udder infections or shedding of the organism via the udder. The second way would be indirect contamination during unsanitary milking practices if the organism were present on the udder surfaces, in feeds, feces, or other environmental sources such as the bedding (Fedio and Jackson, 1992).

Farmyard soil

There are several diverse sources of *L. monocytogenes* present in the dairy farm environments. One source is soil, which receives decaying vegetation, pasture herbage, animal waste, and sewage sludge, all which harbor *L. monocytogenes* (Ryser and Marth, 1999). Because of the ubiquitous, saprophytic nature of *L. monocytogenes*, plant material seems to be an important ecological niche and has been cited as a reservoir for this organism. Farmyard soil samples (n=36) where ewes were raised was contaminated by several *Listeria* spp. at varying rates, which included 5.6% for *L. grayi*, 80.6% for *L. innocua*, and 8.3% for *L. monocytogenes* (Garcia et al., 1996). This author also indicated that three samples were contaminated by both *L. innocua* and *L. monocytogenes*, and two samples by both *L. grayi* and *L. innocua* (Garcia et al., 1996).

Manure

A study of 79 fecal samples taken directly from the rectum or from an observed freshly excreted cow feces showed that 52% tested positive for *L. monocytogenes*, while 67% were positive for *Listeria* spp. (Skovgaard and Morgen, 1988). According to Husu (1990) prevalence of *Listeria* spp. and *L. monocytogenes* from 3,878 fecal samples were higher during the indoor season (12.7% and 9.2% respectively) than in specimens collected when the animals were grazing (5.3% and 3.1%, respectively). There was a strong positive correlation between the presence of *L. monocytogenes* and whether silage was used for feed. Husu (1990) also noted that seasonal variation of *Listeria* in raw milk correlated with the occurrences of *Listeria* in fecal samples rather than to the presence of this organism in grass silage (22.7% and 16.0% samples positive for *Listeria* spp. and *L. monocytogenes*, respectively) or in pasture grass (64.7% and 38.2% samples positive for *Listeria* spp. and *L. monocytogenes*, respectively). In another study, 11.9% of 59 fecal samples examined were positive for *Listeria* spp. Of these samples, 6.8% were *L. monocytogenes* and 10.2% were *L. innocua* (Husu et al., 1990). Van Renterghem et al. (1991) isolated *L. monocytogenes* from fresh cattle feces (20%), but did not isolate *L. monocytogenes* from manured soil samples.

Fenlon et al. (1996) studied the excretion of *L. monocytogenes* by farm animals and the effects of transportation of these animals on excretion. When live animals were delivered to three abattoirs, Fenlon and others (1996) found the level of excretion of *Listeria* in the feces of cattle was greater when animals were transported over long distances (>100 km) as opposed to being transported to nearby abattoirs (<25 km). Monitoring two groups of cattle, Fenlon et al. (1996) demonstrated that while both groups of cattle were grazing, they excreted no *L. monocytogenes*. After silage feeding began, 4 of 14 (28.6%) in one group and 4 of 13 (30.8%) in the second group began excreting *L. monocytogenes*. The numbers of *Listeria* enumerated were low, varying from present in 25g to 11 CFU/g. Further, examination of fecal material of sheep fed a diet of hay

after transport showed no detectable *Listeria*. Fenlon et al. (1986a) suggested that the moisture level in hay was too low to support the growth of *Listeria*.

Prolonged survival of *L. monocytogenes* in fecal material, soil, and grass can lead to the perpetuation of the infective process, especially when animals are housed together in barns over the winter months. Once an asymptomatic animal begins shedding *L. monocytogenes* in feces, the organism is likely to spread rapidly to other animals that are located in close proximity to the shedding animal via various sources such as bedding material (Ryser and Marth, 1999). Once cattle are returned to pasture during the spring, the infectious cycle weakens, because the animals have a decreasing likelihood of coming in contact with infected materials.

Bedding

Garcia and associates (1996) demonstrated the ability to isolate several species of *Listeria* from ewe's bedding. Isolation rates from a total of 44 bedding samples showed that 2.3% were positive for *L. grayi*, 29.5% contained *L. innocua*, and 11.4% harbored *L. monocytogenes*. The authors also indicated that two samples were contaminated by both *L. innocua* and *L. monocytogenes*, and one sample by both *L. grayi* and *L. innocua*.

Airborne

Airborne pathogens originate from sources such as plants, soil, animals, humans, water, or food. Dust particles serve as vehicles for the airborne transmission of microorganisms. Bacteria adhere to dust particle surfaces and may be present in the air in high numbers when dust particles are airborne in the environment (Prescott et al., 1996). A 1987 survey sponsored by the American Meat Institute showed that *Listeria* spp. are widely distributed within the environment of many meat processing plants (Gorham, 1991). *Listeria* spp. was found in compressed air of post-heat processing areas in 4% of 41 meat-processing plants examined (Gorham, 1991). According to Ryser and Marth (1999), the air supply within the food-processing factory must be

considered as a potential source of *Listeria*. Therefore, regular cleaning and repairing of ducts and air filters to remove excessive dust and dirt is recommended.

Insects

Insects have long been known to be vectors of disease. Grebenyuk and colleagues (1981) found *L. monocytogenes* in some migratory birds and in the insects that inhabit their nests. During the course of their research, they isolated *L. monocytogenes* from ixodid ticks, mites, fleas, lice, and larvae of fleas and flies. Gorham (1991) isolated *L. monocytogenes* in ants present in cheese factories in California. Numerous studies have shown that *L. monocytogenes* can remain in the body of ticks for a period of 40 days to one year or longer depending upon the developmental stage of the tick. These studies have demonstrated that *L. monocytogenes* can be transmitted to healthy animals via a bite from a tick infected with *L. monocytogenes* (Grebenyuk et al., 1972; Levina et al., 1983).

Domenichini and associates (1992) examined the role of insects in the transmission of *Listeria*. House flies (*Fannia canicularis*) and beetles (*Dermestes peruvianus* Castelnau), which are frequently found in the food industry, on the farm, and in the human environment were utilized. The flies were dewinged and exposed to *L. innocua* in Petri dishes by crawling over the surface of the inoculated agar. The beetles were fed a liquid meat mixture inoculated with *L. innocua*. Microbiological testing showed that *L. innocua* was excreted in the feces of the beetles. Examination under a scanning electron microscope determined that the *L. innocua* adhered to numerous epidermal structures of the fly. The researchers concluded that insects are both a reservoir and a vector of *Listeria* spp. and thus have the capability to transmit *Listeria* spp. by their presence on domestic and wild animals as well as food sources. These researchers suggest that, even if control measures exterminate insects, prolonged contamination of food sources may still occur due to viable pathogens remaining in and on dead insect carcasses (Domenichini et al., 1992).

EPIDEMIOLOGICAL SURVEILLANCE FOR *LISTERIA MONOCYTOGENES*

Since the 1980's, conventional microbiological techniques developed for detecting and enumerating *Listeria* include a wide range of currently available selective enrichment, and plating media. These techniques do not rapidly yield results to insure the microbiological safety of perishable food products before consumption (Ryser and Marth, 1999). The promulgation of the "zero-tolerance" policy for ready-to-eat foods has driven the development of rapid tests for the detection of *Listeria monocytogenes* from various food products (Ryser and Marth, 1999). The use of advanced molecular fingerprinting techniques has aided in identifying sources and strains of *L. monocytogenes* as well as to narrow the scope of epidemiological investigations. *Listeria* isolates recovered from raw bulk tank milks yielded different random amplified polymorphic DNA (RAPD) profiles (Lawrence et al., 1993) and varying plasmid profiles (Fistrovici and Collins-Thompson, 1990) suggesting that a persistent influx of a variety of *Listeria* strains from the farm environment into bulk tanks occurs over time (Sanaa et al., 1993). Molecular typing methods have been used successfully to trace the transmission of human listeriosis back to foods of animal origin (meat and dairy products), to verify epidemiological associations between silage consumption, to diagnose cattle and sheep listeriosis, to determine shedding of *L. monocytogenes* in raw milk, and to compare a select number of *Listeria* isolates from food processing environments (Batt, 1997). These techniques include pyrolysis mass spectrometry (Low et al., 1992), restriction enzyme analysis (Wesley and Ashton, 1991), multilocus enzyme electrophoresis (Boerlin and Piffaretti, 1991; Graves et al., 1994), RAPD (Czajka and Batt, 1994; Lawrence et al., 1993; Farber and Addison, 1994), pulsed-field gel electrophoresis (Louie et al., 1996), arbitrarily primed PCR (Louie et al., 1996), non-automated ribotyping (Louie et al., 1996; Graves et al., 1994; Swaminathan et al., 1996), and automated ribotyping (Wiedmann et al., 1997a; Wiedmann et al., 1997b; Arimi et al., 1997; Gendel and Ulaszek, 2000; Ryser et al., 1996).

Several of the genomic typing applications, which include non-automated ribotyping, are time consuming, laborious, and sometimes produce data that is unclear. An established automated ribotyping technique has been developed (Qualicon Riboprinter Microbial Characterization System, E. I. du Pont de Nemours and Co. Inc., Wilmington, DE) (Bruce et al., 1995; Hubner et al., 1995), which will identify the selected *Listeria* strains and other bacterial pathogens to the species level, provide discrimination beyond the species level, and characterize the strains via a six-stage automated process. This procedure involves lysing bacterial cells, performing a restriction enzyme (*EcoRI*, *PvuII*, or *PstI*) digestion of the chromosomal DNA, separating the restriction fragments by agarose gel electrophoresis, transferring the fragments to a nylon membrane, and performing a Southern Blot hybridization via a chemiluminescent ribosomal probe (*E.coli rrnB* rRNA operon) of the nylon membrane. A photograph of the luminescent DNA fragment pattern image was obtained using a charge-couple device, a semiconductor device used as an optical sensor that stores charge and transfers it sequentially to an amplifier and subsequently to a detector, charged-coupled device (CCD) camera from the agarose gel, and data processing of the digital image. A genetic fingerprint of riboprint patterns produced based upon generation of rRNA restriction fragment length polymorphisms (RFLP) using a probe that hybridizes to an array of rRNA operons located on various restriction fragments. Riboprint patterns are identified, compared to a library of more than 50 pattern images, and classified according to the software of the riboprinter system (Bruce et al., 1995).

Employing the use of the automated riboprinter with the restriction enzymes *EcoRI* and *PvuII*, Gendel and Ulaszek (2000) compared patterns of 72 isolates of *L. monocytogenes* from smoked salmon samples to the DuPont pattern library. Among the 72 isolates, they found that 73% of the 11 *EcoRI* ribogroups identified were present in isolates collected over a period of >12 months and 75% were found in multiple smoked salmon samples distributed nationally. This made it possible to characterize the geographical and temporal patterns of strain distribution of *L.*

monocytogenes. Of the 72 isolates obtained, 36% of the smoked salmon samples contained more than one isolate. These co-isolates yielded, different ribotype patterns in 35% of the isolates, demonstrating that multiple strains of *L. monocytogenes* can coexist in the same environment (Gendel and Ulaszek, 2000). Several authors stress the importance of picking a sufficient number of colonies per sample when attempting to isolate specific *L. monocytogenes* strains during investigations of listeriosis (Gendel and Ulaszek, 2000; Ryser et al., 1996).

To further understand the epidemiology and pathogenesis of animal listeriosis, *L. monocytogenes* strains isolated from clinical samples during four epizootic listeriosis outbreaks were subjected to automated ribotyping by Wiedmann and associates (1996). Matching ribotypes were found in three of the four outbreaks between silage and clinical isolates, which furthers the longstanding association between the feeding of contaminated silage and outbreaks of listeriosis in ruminants (Wiedmann et al., 1996). A high frequency of the ribotype (RT) dd 1151 was found in the silage isolates tested. At the time this study in 1996 was reported, 2.3% of the isolates (out of 1,346 *L. monocytogenes*) in the DuPont database belonged to this ribotype, indicating that this RT is more commonly associated with specific niches within the agricultural ecosystem, such as silage. Further, ribotype (RT) dd 0566 is present in 19.1% of the strains in the DuPont database. Wiedmann et al. (1996) isolated this RT from 38.8% of the clinical isolates from ruminants, indicating that this particular strain has greater virulence for ruminants than other ribotypes and may be more likely to cause disease than other strains of *L. monocytogenes* residing in farm environments. Ribotyping a large number of veterinary clinical and silage isolates is needed to statistically confirm data regarding the occurrence of various ribotypes (Wiedmann et al., 1996).

Continual development of more advanced genotypic and phenotypic subtyping techniques for characterizing *Listeria* beyond the species level is necessary if reservoirs of the *Listeria* spp. in processed dairy products are to be traced back to the farm environment. Arimi et al. (1997) identified 37 distinct *Listeria* ribotypes (RTs) out of a total of 388 *Listeria* isolates

from 20 different dairy processing facilities. In addition, 44 silage, 14 raw bulk tank milk, and 29 dairy cattle (including, 26 udder quarter milk, 1 brain, 1 liver, and 1 aborted fetus) isolates were included for a total of 475 isolates in this 10-year study. Of these 475 isolates, 93 were *L. monocytogenes* isolates. Sixteen *L. monocytogenes* RTs, including five known RTs responsible for foodborne listeriosis, were identified. These five clinical RTs included 1 RT specific to dairy processing plants, 2 RTs common to dairy processing plants and silage, 1 RT common to dairy processing environments, silage, and dairy cattle with the last RT emerging in dairy processing plants, silage, raw milk bulk tanks, and dairy cattle. Through the use of automated ribotyping, the association between *Listeria* contamination of the farm and the ensuing contamination of the dairy-processing environment can be made. In addition to the 16 *L. monocytogenes* RTs found, 12 *L. innocua*, 5 *L. welshimeri*, 2 *L. seeligeri*, 1 *L. ivanovii*, and 1 *L. grayi* were also found (Arimi et al., 1997). Silage was identified as a reservoir for 4 of 5 clinically significant *L. monocytogenes* ribotypes. *L. innocua* RT 19094 identified in this investigation was found to be abundant in dairy plants and farms and was also recovered by these researchers in raw milk and cheese in the Azores, Vermont. Because of its commonality, *L. innocua* RT 19094 has little epidemiological significance in tracking sources of contamination in dairy processing plants. The *L. monocytogenes* RT 19071 from an infected dairy herd was identified, which suggests contamination specific to the farm environment was dominant among bulk tank milk samples (Arimi et al., 1997).

Recent findings by Ryser and others (1997), have called to question the safety of high-quality (pH <4.0) corn silage. *Listeria* spp. were isolated from 10% corn silage samples, 28% hay silage samples, and 60% grass silage samples. Automated ribotype analysis was performed on these isolates in order to draw comparisons between the incidence of *Listeria* RTs and silage pH. Of the corn silage isolates 3 were *L. monocytogenes* (three RTs, one known clinical RT) and 10 were *L. innocua* (four RTs). Two RTs from two *L. monocytogenes* isolates and three RTs

from 19 *L. innocua* isolates were identified from 21 positive hay silage samples. Corn silage having a pH <4.0 (83% of the samples) was regarded as high quality and contained 8 of the 13 *Listeria* spp. isolates. One of these was a clinical ribotype of *L. monocytogenes* that was linked to human cases of listeriosis caused by consumption of cream in England. Hay silage having a pH $\geq$ 4.0 (92% of the samples) was considered low-quality and contained 20 of the 21 *Listeria* spp. isolated, two of which were non-clinical *L. monocytogenes* RTs (Ryser et al., 1997). Screening for poor-quality (pH $\geq$ 4.0), properly fermented (pH<4.0), and spoiled hay silage would sharply decrease incidences of livestock listeriosis and should be integrated as part of an on-farm hazard analysis critical control point (HACCP) program. The presence of *L. monocytogenes* strains in silage could lead to increased chemical or thermal resistance, increased virulence of strains, and acid-adaptation of cells which may pose a threat to the dairy and meat industries in the future (Ryser et al., 1997).

To aid in an investigation of an epizootic case of listerial encephalitis in sheep in New York State, Wiedmann et al. (1997a) used automated ribotyping to identify contaminated corn silage as the source most likely to be responsible for listeriosis in seven sheep. The same ribotype pattern (dd 0653) was isolated from the silage and the afflicted sheep brains. The investigators were told that the sheep were not fed silage; they were fed haylage, which did contain isolates of *L. monocytogenes* pattern type dd 0566. The authors concluded that corn-silage cross-contaminated the haylage destined for the sheep, via the front-end loader, which was not cleaned between transporting the two different feeds. This was confirmed by showing that all isolates obtained from corn silage residues in the front-end loader and the seven sheep had the same ribopattern. The contaminated corn silage, containing the same strain of *L. monocytogenes* that afflicted the sheep in this study, was fed to dairy cows on a different farm and no listeriosis cases were reported. The ribopattern dd 0653 associated with infected sheep and contaminated silage

in this study has also been responsible for a food-borne epidemic of listeriosis in humans in Massachusetts in 1983 (Wiedmann et al., 1997a).

Arimi and others (1997) reported that *Listeria innocua* RT 19094 was frequently present in dairy processing facilities and farms. The same ribotype was also reported to be commonly associated with raw retail meat and poultry products (Ryser et al., 1996). The presence of other *Listeria* strains, which include *L. innocua*, can out-compete *L. monocytogenes* during enrichment (Curiale and Lewus, 1994; Petram and Swanson, 1993). Accurate *Listeria* enrichment, isolation methods, and continued subtyping of multiple *L. monocytogenes* strains from suspected silage will enhance the recognition of silage-related listeriosis outbreaks in dairy cattle (Arimi et al., 1997). A wide range of *Listeria* electrophoretic types are present at the primary production environment. Virulent strains do arise at the farm level, which occasionally contaminate raw food material, and can adapt themselves to the food-processing environment. This occurrence may lead to more widespread contamination of food products available to the consumer (Fenlon et al., 1996).

PURPOSE OF CURRENT RESEARCH

It seems very likely that the original source of processing plant contamination with *L. monocytogenes* may be the farms that generate the raw product (Pritchard et al., 1994). Findings by Arimi and associates (1997), that support the link between on-farm sources of *Listeria* contamination (dairy cattle, raw milk, silage) and subsequent contamination of dairy processing environments, stress the importance of farm-based HACCP (Hazard Analysis Critical Control Points) programs for controlling listeriae. Adequate research data on *Listeria* contamination, that is needed to develop on-farm pathogen-control programs, is unavailable (Cullor, 1995). Such data is essential for risk assessment and risk management decisions. Once data is available for program development, applying a proactive pathogen-reduction approach to farms producing milk and beef will address on-farm food safety concerns.

Although there has been previous research conducted on the detection of *Listeria* spp. via analysis of bodily secretions (Husu, 1990; Husu et al., 1990; Fedio and Jackson, 1992; Arimi et al., 1997; Skovgaard and Morgen, 1988; Van Renterghem et al., 1991; Fenlon et al., 1996; Pritchard et al., 1994; Rohrbach et al., 1992) or swabbing selective sites of dairy cattle (Husu et al., 1990; Fedio and Jackson, 1992) as well sampling various sites of the dairy farm environment (Husu et al., 1990; Rodriguez et al., 1994; Garcia et al., 1996; Skovgaard and Morgen, 1988; Arimi et al., 1997; Fenlon et al., 1996) research to date has inadequately addressed the optimal recovery (percentage isolation) of *Listeria* spp. and *L. monocytogenes* from dairy cows, calves, and farm environmental sampling sites. This may be due to time constraints and lack of funding for a comprehensive survey of a combination of several cattle and farm environmental sites.

Various studies have demonstrated the usefulness of strain-specific automated ribotype analysis for a variety of epidemiological studies (Ryser et al., 1997; Arimi et al., 1997; Wiedmann et al., 1996), Wiedmann et al., 1997a; Ryser et al., 1996; Gendel and Ulaszek, 2000). Previous research efforts have not focused on the use of automated ribotype analysis of phenotypically confirmed *Listeria* isolates from a variety of sites on the exterior of dairy cows and calves in addition to various environmental locations associated with the farm that are frequented by these animals. Research is needed to develop baseline data that compare *Listeria* ribotypes of isolates from dairy cows and calves and sites on the farm environment to clinical *Listeria* ribotypes associated with animal, human, or foodborne outbreaks of listeriosis.

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PART II

**PHENOTYPIC INCIDENCE OF *LISTERIA* ISOLATES RECOVERED FROM DAIRY
COWS, CALVES, AND THE FARM ENVIRONMENT**

ABSTRACT

The objective of this study was to determine the major sources of *Listeria monocytogenes* and *Listeria* spp. associated with dairy cows, calves, and the farm environment. Isolation and confirmation of *Listeria* spp. was performed according to the Food and Drug Administration's Bacteriological Analytical Manual (AOAC). Modifications in isolation protocol were made to determine the prevalence and optimal isolation media (MOX or PALCAM) for the detection of *L. monocytogenes* (LM) and other *Listeria* spp. from dairy farm cattle and environmental samples. Cows (n=30) (foremilk, hair, oral, rectal, and teats) and calves (n=30) (hair, oral, and rectal) were sampled over a six-month period. The environmental samples (n=20) included bulk tank milk, air, trough water (n=22), feed, bedding, farmyard soil, grain, silage, insects, milking equipment (cups, hoses, inflations), and river water (n=10) adjacent to the dairy farm. LM was more frequently isolated from the dairy farm environment (21.3%) than from dairy cows (18.5%) or calves (14.4%). All dairy cow, calf, and farm environmental sites, except for air, tested positive for the presence of LM. Based on the enrichment and isolation protocols evaluated in this research, the authors recommend that MOX be used for all sample types associated with dairy farms with the exception of soil, rectal, and trough water in which PALCAM gives better isolation of *L. monocytogenes*. The development of a stepwise "on-farm" pathogen reduction program to control LM in dairy cows and calves will be a challenge due to the frequent occurrence of LM in the environment of dairy cows. Protocols for sampling should be selected which can consistently detect the presence of LM.

INTRODUCTION

In the early 1980's, Human listeriosis was first associated with the consumption of coleslaw that was traced back to a farm where sheep manure was used to fertilize the cabbage fields (Schlech et al., 1983). Since then, listeriosis has been associated with pasteurized milk (Fleming et al., 1985), unpasteurized milk (Dalton et al., 1997), soft cheeses (Bille, 1990; Linnan et al., 1988), hot dogs (Barnes et al., 1989) and deli meats (Anonymous, 1999). Product recalls (Ryser and Marth, 1999) and multi-million dollar lawsuits suffered by the dairy and meat industries have resulted in a "zero-tolerance" policy for the presence of *Listeria monocytogenes* in and on all cooked/ready-to-eat foods and dairy products.

Previous research in various countries has found *L. monocytogenes* in raw cows' milk with a distribution ranging from approximately 0.1 to 45% (Ryser and Marth, 1999). A 23-year survey of cows (n=1,132,580) quarter milk samples showed that the percentage of cows infected with *L. monocytogenes* ranged from 0.01 to 0.1% (Jensen et al., 1996). Herds (n=36,199), with a *Listeria* infected cow, showed a low, but constant level of infection (0.2 to 4.2%), during the study (Jensen et al., 1996). Recovery of *Listeria* isolates from raw bulk tank milk suggests that a constant influx of diverse *Listeria* strains from the farm environment into bulk tanks occur over time (Sanaa et al., 1993). *L. monocytogenes* can be shed in the milk of apparently healthy dairy cows (Husu, 1990; Skovgaard and Morgen, 1988) and thus may pose a threat to public health (Ryser and Marth, 1999).

Listeriosis in dairy cows and livestock can occur sporadically or as an epidemic and results in *Listeria*-related mastitis, abortion, or encephalitis (Ryser and Marth, 1999). Dairy cattle can also asymptotically shed *Listeria monocytogenes* in their feces (Fenlon et al., 1996; Hofer, 1983) as well as in their milk (Hird and Genigeorgis, 1990). Once an asymptomatic animal begins shedding *L. monocytogenes* in feces, the organism is likely to spread rapidly to other animals that are located in close proximity to the shedding animal via various sources such as

bedding material (Ryser and Marth, 1999). Contamination of bulk milk may occur in one of two ways: directly, as a result of udder infections or shedding of the organism via the udder or indirect contamination during unsanitary milking practices (Fedio and Jackson, 1992).

There are several diverse sources of *L. monocytogenes* present in the dairy farm environment which includes agricultural farmland soil that receives decaying vegetation, pasture herbage, animal waste, and sewage sludge (Ryser and Marth, 1999). Water run-off from the agricultural ecosystem, as well as the increasing use of land-bound waterways for recreation, transportation, and industrial effluents (such as from sewage treatment plants) may result in the increasing presence of *L. monocytogenes* in lakes, rivers, and streams. Arumugaswamy and Gibson (1999) suggested that *Listeria* positive samples from the Windsor River, in New South Wales (Australia), were due to (the number of outfalls) from farmlands located along its banks.

Listeria monocytogenes has previously been found in migratory birds and in the insects that inhabit their nests, such as, ixodid ticks, mites, fleas, lice, larvae of fleas and flies (Grebenyuk et al., 1981). *L. monocytogenes* was isolated from ants in cheese factories in California (Gorham, 1991). Researchers maintain that even if control measures exterminate insects, prolonged contamination of food sources may still occur due to viable pathogens remaining in and on dead insect carcasses (Domenichini et al., 1992). Gray (1960b) proposes that many healthy wild and domestic animals may be carriers of *L. monocytogenes* and may shed it in normal body discharges.

Improper processing, fermentation, and management of silage may play a role in the transmission of listeriosis to ruminants as well as serving as a source for *Listeria* spp. on dairy farms (Fenlon, 1986b; Gray, 1960a; Ryser and Marth, 1999; Vazquez-Boland et al., 1992). More recently, the safety of high-quality (pH <4.0) corn silage has been called into question (Ryser et al., 1997). It seems very likely that the original source of processing plant contamination with *L. monocytogenes* may be the farms that generate the raw product (Pritchard et al., 1994). Research

data on microbial contamination at the farm, which is needed to develop on-farm pathogen-control programs, is still inadequate (Cullor, 1995).

Therefore, the objective of this study was to determine the occurrence of *L. monocytogenes* and *Listeria* spp. among dairy cows, calves, and environmental reservoirs. A secondary objective was to compare MOX and PALCAM agar to determine the optimal recovery of *L. monocytogenes* from dairy cows, calves, and environmental sites.

MATERIALS AND METHODS

Animal sampling

Swab samples (n=2 per sampling site) were taken from the teat, rectum, oral cavity, and hair from the back of the neck (20cm X 20cm area) of cows (n=30) and calves (n=30) at the University of Tennessee Dairy Farm (Knoxville Experimental Station) over a six-month period. One cow and one calf were sampled every 12 days. Prior to sampling each animal, sterile cotton tipped swabs (2 per sampling area) were dipped in Butterfield's Phosphate Buffer (BPB), 0.01 M, pH 7.2. Samples were taken and immediately placed into individually labeled test tubes containing 9 ml of *Listeria* Enrichment Broth (LEB) (Difco Laboratories, Detroit, MI). Foremilk, (first 4 squirts) from teats of lactating cows were collected into sterile Whirl-pak™ plastic bags (Nasco, Ft. Atkinson, WI). Samples were transported to the microbiology lab for analysis within two hours of sampling. Foremilk samples (1 ml) were inoculated into 9 ml of *Listeria* Enrichment Broth (LEB) (Difco). It is important to note that during the sampling period (11/99-5/01) this dairy herd of 320 cattle has had intermittent episodes of mastitis. However, listeric mastitis is rare and the mastitis in the herd is primarily due to *Staphylococcus aureus*, *Streptococcus* spp. (primarily *S. uberis*), and occasionally gram-negative organisms (Oliver, 2001).

Environmental sampling

Air samples (n=20) were collected every 36 days over a 12-month period at the University of Tennessee Dairy Farm from the barn housing dairy calves as well as from the milking parlor. Sampling was performed by venting air through micro-filters (Millipore™, 0.45µm), using a Gast vacuum pump, model 0322-V4BG18DX (Gast Co., Benton, MI). The pump was connected to a Gilmont flow meter, model 65 (Gilmont Instruments, Barrington, IL). The vacuum line was split into two outlets from the flow meter, connected to two inlets, and attached to two sterile vacuum flasks. Each flask was fitted with a Nalgene Analytical Filter Funnel (type AF, Cat. No. 140) (Nalgene Nunc International Corp., Rochester, NY) containing a bacteriological micro-filter (Millipore™, 0.45µm). Before sampling, micro-filters were moistened with BPB, 0.01 M, pH 7.2. Airflow was set at 37.56 SLPM (standard liters per minute) and the pump connected to an automatic timer set for 20 minutes (750 liters). The micro-filters were aseptically placed into sterile small Whirl-pak™ plastic bags (Nasco, Millville, NJ) using sterile forceps and transported to the microbiology lab for analysis within one hour of sampling. A 9 ml aliquot of LEB was added to each bag containing a micro-filter.

Bulk tank milk (n=20) and trough water samples (n=22) were collected every 36 days over a 12-month period in separate sterile 250 ml Wheaton™ bottles, placed in a cooler, and transported to the microbiology lab for analysis within fifteen minutes of sampling. Bulk tank milk (25ml) and trough water (25ml) were inoculated into separate Stomacher bags containing 225 ml of LEB. Bulk tank milk was taken from the bulk tank located in a separate section of the milking parlor. Water samples were obtained from drinking troughs of the maternity (cows in gestation), dry cow (not producing milk), calf, and lactating (producing milk) barns located on the University of Tennessee Dairy Farm.

Bulk samples (n=20), of feedbunk (a mixture of hay, silage, and grain), bedding, pasture soil, as well as grain and silage (obtained from separate vertical silos) were collected every 36

days over a 12-month period in separate one-gallon Ziploc™ bags and transported to the microbiology lab for analysis. To obtain the pH of silage, the methodology outlined by Perry and Donnelly (1990) was utilized. A 25 g portion of silage from the bulk sample was placed into a sterile bag containing 225 ml of deionized water and allowed to equilibrate at room temperature for 15 min. The pH was measured and recorded for each silage sample using an Accumet Basic pH meter (Fisher Scientific, Pittsburgh, PA). Twenty-five gram portions of each bulk sample were placed into separate sterile bags each containing 225 ml of LEB. Bulk samples were collected from the barns and surrounding habitats of lactating cows, dry cows, maternity cows, and calves on the University of Tennessee Dairy Farm.

Water samples were collected in sterile bottles (1000 ml) from a portion of the Tennessee River that runs adjacent to the University of Tennessee Dairy Farm. In April and May of 2001, samples (n=10) from each of three sites were taken at the riverbanks from the beginning, midpoint, and the end of the farm. Samples from each site were immediately transported to the microbiology laboratory for analysis. Samples (100 ml) from each site were vacuum filtered through three separate plastic disposable Nalgene Analytical Filter Funnels (type AF, Cat. No. 140) (Nalgene Nunc), each containing a micro-filter (Millipore™, 0.45 µm). Each micro-filter was aseptically placed into three separate, sterile, plastic bags using sterile forceps. A 9 ml aliquot of LEB was added to each bag containing a micro-filter.

Swab samples (n=20 for each milking component) of the milking equipment (Alfa-DeLaval, Kansas City, MO) were taken every 36 days over a 12-month period from various components of the milking apparatus in the milking parlor. The inflations (apparatus attaching to each of the teats of lactating cows), the cup (apparatus where the milk is sucked into from the inflations), and the hoses (leading away from the cup and to the pipe that carries milk to the bulk tank) were swabbed. The swabs were immediately placed into separate test tubes, each containing 9 ml of LEB and transported to the microbiology lab for analysis within one hour of

sampling. Insect samples (n=20) were collected from the calf, sick cow, and lactating cow barns only during the late spring, summer, and early fall months from the University of Tennessee Dairy Farm. Due to climate changes, there were insufficient amounts of insects during the late fall, winter, and early spring months. A minimum of three Terro™ fly catchers (Senaret Chemical Co., St. Louis, MO) were placed in representative locations within the various barns 48 hours to seven days before collection of the insects. When sufficient insects were obtained, the fly catchers were taken down, put into one-gallon bags, and transported to the microbiology laboratory for analysis. Approximately 1 g of the fly ribbon containing the insects was placed into a labeled small sterile plastic bag containing 9 ml of LEB.

Enrichment, isolation, and purification

Animal and environmental samples were analyzed for the presence of *L. monocytogenes* according to the FDA Bacteriological Analytical Manual (BAM) (Hitchins, 1998). Isolation and biochemical confirmation protocols were modified for samples not covered in BAM. Sample cultures were mixed (swabs samples) or pummeled (environmental solid samples) for 2 min using a stomacher (Stomacher 400 Seward Laboratory Blender, Tekmar, Cincinnati, OH), pH was adjusted to 7.3 ± 0.2 at 25°C using an Accumet Basic™ pH meter (Fisher Scientific, Pittsburgh, PA), and cultures were incubated for enrichment at 30°C for 48 h. To avoid cross-contamination of samples, the probe of the pH meter was sanitized with a 10% hypochlorite (Clorox™ Co., Oakland, CA) solution and subsequently rinsed with deionized water prior to taking the pH of each sample. Media were prepared according to manufacturer's instructions, including the addition of selective supplements per instructions for preparation of PALCAM (Difco Laboratories, Detroit, MI) (Difco: ceftazidime, 40 mg) and modified Oxford medium (MOX) (Difco Laboratories, Detroit, MI) (Difco: colistin sulfate, 10 mg; moxalactam, 20 mg). Enriched samples were streaked for isolation onto MOX and PALCAM media and incubated aerobically at 35°C for 48 h. Suspect (typical and atypical) colonies from both media were picked and re-

streaked for purification onto both selective media (MOX and PALCAM) and incubated aerobically at 35°C for 48 h. Presumptive *Listeria* colonies from both media were picked and streaked for isolation onto separate tryptic soy agar (TSA) (Difco) slants and incubated aerobically at 35°C for 48 h.

Biochemical confirmation

L. monocytogenes and *Listeria* spp. were confirmed by the following biochemical tests: catalase production, motility growth at 25°C and 37°C utilizing motility medium S (Difco) with the addition of 1% 2, 3, 5 – triphenyltetrazoliumchloride (Sigma Chemical Co., St. Louis, MO), beta-hemolysis (5% sheep blood agar). The CAMP (Christie Atkins Munch-Peterson) test (McKellar, 1994) (5% sheep blood agar) were performed on each isolate in the presence of *Staphylococcus aureus* ATCC 49444 (American Type Culture Collection, Rockville, MD). Blood agar plates (Difco) (beta-hemolysis and CAMP tests) were incubated at 35°C and checked for hemolysis at 24 and 48h. The motility medium S was read for the presence/absence of motility after 48 h and at 7 days. Tumbling motility was evaluated microscopically. Isolates were inoculated into tubes containing 5 ml Purple Broth Base (Difco) with each of the following carbohydrates at 1.0%: mannitol (Difco), xylose (Difco), and rhamnose (Difco). Filter-sterilized xylose was aseptically added after autoclaving the broth base (Pritchard et al., 1994). The sugars were read for the presence/absence of a color change from purple to yellow after 48 h and 7 days at 35°C.

Statistical Analysis

The presence/absence of *L. monocytogenes* or other *Listeria* spp. was determined from the biochemical tests and recorded for each cow, calf, and farm environmental site. Data were calculated as isolation percentages and categorized as either: colonies not detected, not *Listeria*, *L. monocytogenes*, or other *Listeria* spp. An analysis of variance (ANOVA) was conducted to determine differences between the isolation percentages of the four designated categories for each

sampling location on cows, calves, and the dairy farm environment (SAS, 1999). A Duncan's multiple range test for the calf data and a Least Squared Means with a Dunnett test (SAS, 1999) was performed for both cow and environmental unbalanced data as part of the ANOVA (SAS, 1999). A multivariate ANOVA with a Wilks-Lambda test was carried out to determine differences between the media (MOX and PALCAM) isolation percentages (SAS, 1999). A multiple linear regression analysis was performed to evaluate the differences in the isolation percentages between MOX and PALCAM of the four designated categories for each sampling site among cows, calves, and the dairy farm environment (SAS, 1999).

RESULTS AND DISCUSSION

Isolation of *L. monocytogenes* from dairy cows, calves, and the environment

L. monocytogenes was more frequently isolated from the dairy farm environment (21.3%) than from dairy cows (18.5%) or calves (14.4%) (Table 1). *Listeria* spp., not *L. monocytogenes*, were more consistently detected from dairy calves than from dairy cows or the farm environment, with cows being more positive for *Listeria* spp. than the farm environment (Table 1). All cow, calf, and environmental isolates were positive for *Listeria* spp. or *L. monocytogenes* (Table 1).

As shown in Table 2, the detection of *L. monocytogenes* in raw bulk tank milk in this study reached 30%, which is consistent with results obtained in other studies (0.1% to 45%) reported by Ryser and Marth (1999) and with other studies utilizing different enrichment and/or isolation media (Rohrbach et al., 1992; Fenlon and Wilson, 1989; Wnorowski 1990; Hayes et al., 1986; Rea et al., 1992; Desmaures et al., 1997; Steele et al., 1997). In the present study, *L. monocytogenes* was detected on all three sites of the milking equipment, which includes the inflations (10%), cups (30%), and the hoses (20%). *L. monocytogenes* was also isolated from samples taken from the bulk tank (30%), and from the foremilk (20%) of cows, while *L. innocua* was isolated from the bulk tank (10%), cups (30%), and inflations (20%) (Table 2). Of raw milk

Table 1. *Listeria* spp. isolated from Dairy Cows, Calves, and the Farm Environment

Species	Sites (% Positive)		
	Cows (n=130)	Calves (n=90)	Farm Environment (n=272)
<i>L. monocytogenes</i> (LM)	18.5	14.4	21.3
<i>Listeria</i> spp. not LM	65.4	65.5	67.6
Negative for <i>Listeria</i>	16.1	20.1	11.1

Table 2. Prevalence of *Listeria* spp. in Raw Milk and on Milking Equipment (n=10 for each site except foremilk)

Species	Sites (% Positive)				
	Bulk Tank Milk	Cups ^a	Foremilk ^b (n=10)	Hose ^c	Inflations ^d
<i>L. grayi</i>	ND ^e	10	ND	10	10
<i>L. innocua</i>	10	30	ND	ND	20
<i>L. ivanovii</i>	ND	ND	ND	ND	10
<i>L. monocytogenes</i>	30	30	20	20	10
<i>L. seeligeri</i>	20	40	20	60	50
<i>L. welshimeri</i>	10	ND	ND	ND	10
<i>L. spp.</i> ^f	50	20	40	20	30

^a Cups: Central area where milk is sucked into from the inflation

^b Foremilk: First squirts from teats of lactating cows

^c Hoses: Hoses leading away from the cup and to the pipe that carries milk to the bulk tank

^d Inflations: Apparati attaching to each of the teats of lactating cows

^e Not detected

^f "Listeria like" isolates: shared similar biochemical confirmation reactions with two species

and milking equipment samples, the cups of the milking equipment and the bulk tank milk had the highest incidence of *L. monocytogenes*. As illustrated in Table 2, *L. ivanovii* was only isolated from the hoses of the milking equipment. *L. seeligeri* was found in the bulk tank milk, foremilk, as well as the milking equipment cups, inflations, and hoses. *L. seeligeri* was detected in greatest frequency from the milking equipment hoses (Table 2). *Listeria*-like isolates (*Listeria* spp.) were most frequently detected from the bulk tank milk and from the foremilk of cows (Table 2). Using other types of enrichment and/or isolation media, researchers have found ewe's milking equipment (Garcia et al., 1996) and dairy cow milking equipment (Husu et al., 1990) to be free of contamination by all *Listeria* spp. In a one-year study involving weekly analysis of the right hindquarter of a Simmental cow, listerial counts of 40,000 CFU/ml of milk were detected during peak lactation with 80,000 CFU/ml from the foremilk alone (Wagner et al., 2000).

Of the feeds and trough water sampled (Table 3), the highest incidence of *L. monocytogenes* was from the feedbunk (80%), which is a mixture of hay, silage, and grain. The second greatest isolation frequency of *L. monocytogenes* was from the silage (60%) (Table 3). The pH silage in this study ranged from 3.75 to 4.86. A survey of seven dairy farms in Jutland, Denmark (Skovgaard and Morgen, 1988) found the occurrence of *L. monocytogenes* in silage (pH>4.5), silage ($\leq$ 4.5), NH₃-treated (alkaline treated) straw, and other dairy cattle feed (hay, mash, and beet debris) to be 63%, 67%, 63%, and 50%, respectively. A subsequent investigation of dairy farms in Finland found that the incidence of *L. monocytogenes* (3.8%) and *Listeria* spp. (10.5%) in silage samples with pH < 4.0 was considerably lower than in samples with pH >4.2 (32.3% and 43.1%, respectively) (Husu et al., 1990).

The semi-stagnant, microenvironment of cow watering troughs that exist within the confines of the farm environment may be visited numerous times a day by dairy cattle and calves that can be considered both reservoirs and carriers of *Listeria*. Through repeated visitations to the

Table 3. Prevalence of *Listeria* spp. in Feeds (n=10) and Trough Water (n=11) from the Dairy Farm

Species	Sites (% Positive)			
	Feedbunk ^a	Grain	Silage	Trough Water
<i>L. grayi</i>	ND ^b	10	ND	ND
<i>L. innocua</i>	40	10	10	9
<i>L. ivanovii</i>	ND	10	30	ND
<i>L. monocytogenes</i>	80	40	60	9
<i>L. seeligeri</i>	80	90	70	36
<i>L. welshimeri</i>	10	10	ND	9
<i>L. spp.</i> ^c	40	70	70	18

^a A mixture of hay, silage, and grain

^b Not detected

^c “*Listeria* like” isolates: shared similar biochemical confirmation reactions with two species

watering trough each day, the potential transmission of listeriosis may occur. The level of *L. monocytogenes* found in cow and calf trough water on the University of Tennessee Dairy Farm (Knoxville Experimental Station) was 9% (Table 3). *L. innocua* and *L. seeligeri* were detected in the feedbunk, grain, silage, and trough water (Table 3). *L. seeligeri* was isolated in the greatest frequency from all feed samples and the trough water. As seen in Table 3, *L. grayi* was isolated only from the grain samples. *Listeria*-like isolates (*Listeria* spp.) were detected in the greatest percentage from the silage and the grain samples. The largest distribution of all *Listeria* spp. isolated from feeds and trough water was from the grain samples (Table 3). Comparatively, in research done by Garcia and colleagues (1996), 51 ewe drinking water trough samples, 31.7% tested positive for *L. innocua* and 7.8% test positive for *L. monocytogenes*. Furthermore, one sample contained both *L. grayi* and *L. innocua*, and in a second sample both *L. innocua* and *L. monocytogenes* were present (Garcia et al., 1996). A high incidence of *L. monocytogenes* (48.0%) and of *Listeria* spp. (52.0%) in dairy cow watering cups has been reported (Husu et al., 1990). Garcia et al. (1996) found that ewe's feeds collected every three months for one year yielded contamination rates of 1.2%, 11.9%, and 1.2% by *L. grayi*, *L. innocua*, and *L. monocytogenes*, respectively. They found that *L. monocytogenes* was present in all concentrate samples, 59% of the hay samples, and was absent from silage samples. *Listeria* spp. was detected in 62.5% of hay samples, 12.5% of silage samples, and below 100 CFU/g in feeds (Garcia et al., 1996).

Feed and water may be contaminated by particles of feed that drop from the animal's face and mouth back into the feeding and watering trough where an environment exists that allows the proliferation of *Listeria* spp. These feeding and watering areas can also be contaminated by the discharge of infectious bodily fluids from the animals' mouths, nares, or conjunctiva during the feeding process. Working in Finland, Husu and others (1990) surmised that the spreading of feeds by farm workers prior to handling milking equipment may result in contamination of milk.

Therefore, the authors recommend that it may be essential to avoid handling of animal feed during or prior to milking. Research by Wiedmann and associates (1997) has suggested that cross-contamination of feeds (i.e., haylage) with tainted silage, by farm equipment used to transport animal foodstuffs may need to be prevented.

In addition to contaminating feeds and watering troughs, bodily secretions infected or colonized with *Listeria* spp. may also contaminate wooden feeding troughs. It has been shown that *L. monocytogenes* is able to survive in and on wood for years. During the 1987 investigation of the outbreak of listeriosis, which continued from 1983 to 1987, where Vacherin Mont d'Or cheese served as the vehicle for infection by *L. monocytogenes*, it was determined that the wooden hoops used to ripen the cheese and the wooden shelves and cheese boxes in the ripening cellars were harboring the epidemic strains of *L. monocytogenes* implicated in the outbreak. As a result of this investigation, all wooden equipment and wooden shelving used to ripen, store, and transport this cheese was removed from the ripening cellars and burned (Bille, 1990; Ryser and Marth, 1999).

The highest isolation frequency for a species from bedding (80%), insect (80%), and soil (80%) samples was *L. seeligeri* (Table 4). *L. monocytogenes* was isolated from bedding (30%), insects (60%), and soil (30%) sites with the greatest contamination rate from insects (60%). *L. innocua* was detected from the bedding, insects, and soil. *L. innocua* was isolated in greatest frequency from the air (50%) and insects (50%). As seen in Table 4, *L. ivanovii* was detected only from soil samples. *L. grayi* was not found in air, bedding, insects, or soil. *L. welshimeri* was only isolated from the bedding (Table 4). There was a significant difference ($P < 0.05$) in the recovery of *Listeria* spp. or *L. monocytogenes* on MOX versus PALCAM among each replication and for each types of environmental sample. The highest prevalence of *Listeria*-like isolates (*Listeria* spp.) was found in air (50%) and insect (60%) samples (Table 4). Garcia and associates (1996) also documented the occurrence of several species of *Listeria* concurrently from

Table 4. Occurrence of *Listeria* spp. from other Dairy Farm Sources (n=10 for each site sampled)

Species	Sites (% Positive)			
	Air	Bedding	Insects	Soil
<i>L. grayi</i>	ND ^a	ND	10	ND
<i>L. innocua</i>	50	20	50	10
<i>L. ivanovii</i>	ND	ND	ND	10
<i>L. monocytogenes</i>	10	30	60	40
<i>L. seeligeri</i>	50	80	80	80
<i>L. welshimeri</i>	ND	20	ND	ND
<i>L. spp.</i> ^b	50	20	60	30

^a Not detected

^b “*Listeria* like” isolates: shared similar biochemical confirmation reactions with two species

frequently contaminated ewe's bedding. Isolation rates from a total of 44 bedding samples showed that 2.3% were positive for *L. grayi*, 29.5% contained *L. innocua*, and 11.4% harbored *L. monocytogenes*. In ewe's farmyard soil samples (n=36) Garcia and others found contamination by several *Listeria* spp. at varying rates, which included 5.6% for *L. grayi*, 80.6% for *L. innocua*, and 8.3% for *L. monocytogenes* (Garcia et al., 1996). Conversely, other studies found farmyard soil free of *L. monocytogenes* (Van Renterghem et al., 1991). Insects have long been known to be vectors of disease. Insects on the farm frequent numerous sites and are able to transport bacteria via epidermal structures on their bodies and excrete environmental bacteria in their feces (Domenichini et al., 1992). Thus, insects have a high potential for the transmission of *Listeria* spp. throughout the farm. As was concluded by Domenichini and associates (1992), insects are both a reservoir and a vector of *Listeria* spp. and have the capability to transmit *Listeria* spp. by their presence on domestic and wild animals as well as human and animal food sources. Our data clearly show that insects are vectors for the transmission of *L. monocytogenes*.

Associated with cows, *L. monocytogenes* was isolated from the foremilk (20%), hair (20%), oral cavities (33%), rectums (20%), and teats (33%) (Table 5). The two highest isolation frequencies of *L. monocytogenes* on cows were from the teat and oral samples. *L. innocua* was detected from the hair (47%), oral cavities (33%), rectums (33%), and teats (40%) of cows. *L. seeligeri* was isolated from foremilk, hair, oral, rectal and teat sites on cows. The most frequent detection of *L. seeligeri* was from the oral cavities and teats of cows. *L. welshimeri* was the least frequently detected species from all sites on cows. There was a significant difference ($P<0.05$) in the recovery of *Listeria* spp. and *L. monocytogenes* on MOX versus PALCAM among the 15 cows, but not among cow sampling sites with the exception of the foremilk. The greatest occurrence of *Listeria*-like isolates (*Listeria* spp.) was from the foremilk samples of cows (Table 5). Husu and colleagues (1990) found that no *Listeria* was detected from the teat swabs of 59 cows taken after washing and drying of cow udders. Prior to cleaning, *Listeria* species were

Table 5. Prevalence of *Listeria* spp. on Cows (n=15 by site except foremilk, n=5)

Species	Sites (% Positive)				
	Foremilk ^a (n=10)	Hair	Oral	Rectal	Teat
<i>L. grayi</i>	ND ^b	20	ND	7	ND
<i>L. innocua</i>	ND	47	33	33	40
<i>L. ivanovii</i>	ND	7	ND	13	7
<i>L. monocytogenes</i>	20	20	33	20	33
<i>L. seeligeri</i>	20	67	73	67	73
<i>L. welshimeri</i>	ND	ND	7	ND	7
<i>L. spp.</i> ^c	40	20	33	7	20

^a Foremilk: First 4 squirts from teats of lactating cows

^b Not detected

^c “*Listeria* like” isolates: shared similar biochemical confirmation reactions with two species

isolated from 30.4% of the cow teat samples taken in the largest barn, and *L. monocytogenes* was detected in 11.1% of the teat samples from cows in the smallest barn (Husu et al., 1990). Fedio and Jackson found a high incidence of *L. monocytogenes* (15.0%), in rectal fecal swabs taken in a 1992 study.

As illustrated in Table 6, *L. monocytogenes* was detected on calves from the hair (20%); however, *L. monocytogenes* were also isolated from the oral cavities (33%) and rectums (13%) of calves. The hair (53%), oral cavities (67%), and rectums (87%) of calves were most often contaminated with *L. seeligeri*. *L. innocua* was isolated at the same frequency from all three sampling sites of calves. The species that were least frequently detected from calves were *L. grayi* and *L. welshimeri* (Table 6). The greatest occurrence of *Listeria*-like isolates (*Listeria* spp.) was from the hair of calves. The most diverse range of *Listeria* detected from calves was from the oral cavity and rectum (Table 6). There was a significant difference ($P < 0.05$) in the recovery of *Listeria* spp. or *L. monocytogenes* on MOX versus PALCAM among the 15 calves, but not among calf sampling sites.

Due to silage and feedbunk consumption by cows versus pellet dried feed consumed by calves, oral and intestinal flora may differ in cows and calves and thus effect the occurrence of *L. monocytogenes* and other *Listeria* at these sites. This may be seen by comparing tables 5 and 6, where *L. monocytogenes* levels in cows that have had a longer exposure to and consumption of silage, reached 33% and 20% at oral cavities and rectums, respectively. *Listeria monocytogenes* levels in calves, which consume pelleted dried feed, were 33% and 13% at oral cavities and rectums, respectively. *L. innocua* levels at the oral cavity and rectums were 33% and 47%, respectively, for both cows and calves.

Table 7 shows that *L. seeligeri* was the most prevalent species isolated from each of the three river water sites (Figure 1) sampled along the University of Tennessee Dairy Farm

Table 6. Prevalence of *Listeria* spp. on Calves (n=15 by site)

Species	Sites (% Positive)		
	Hair	Oral	Rectal
<i>L. grayi</i>	ND ^a	13	ND
<i>L. innocua</i>	47	47	47
<i>L. ivanovii</i>	7	ND	7
<i>L. monocytogenes</i>	20	33	13
<i>L. seeligeri</i>	53	67	87
<i>L. welshimeri</i>	ND	7	7
<i>L. spp.</i> ^b	67	60	53

^a Not detected.

^b "Listeria like" isolates: shared similar biochemical confirmation reactions with two species

Table 7. Occurrence of *Listeria* spp. in Tennessee River Water Adjacent to the Dairy Farm
(n=5 for each site sampled)

Species	River Samples (% Positive)		
	Site 1 (River Baseline) ^a	Site 2 (Middle) ca 0.7 km ^b	Site 3 (End) ca. 2.08 km ^c
<i>L. grayi</i>	ND ^d	ND	ND
<i>L. innocua</i>	20	20	20
<i>L. ivanovii</i>	ND	ND	ND
<i>L. monocytogenes</i>	ND	20	80
<i>L. seeligeri</i>	80	100	80
<i>L. welshimeri</i>	40	ND	ND
<i>L. spp.</i> ^e	60	80	100

^a River water sampling site immediately before the beginning of the farm property

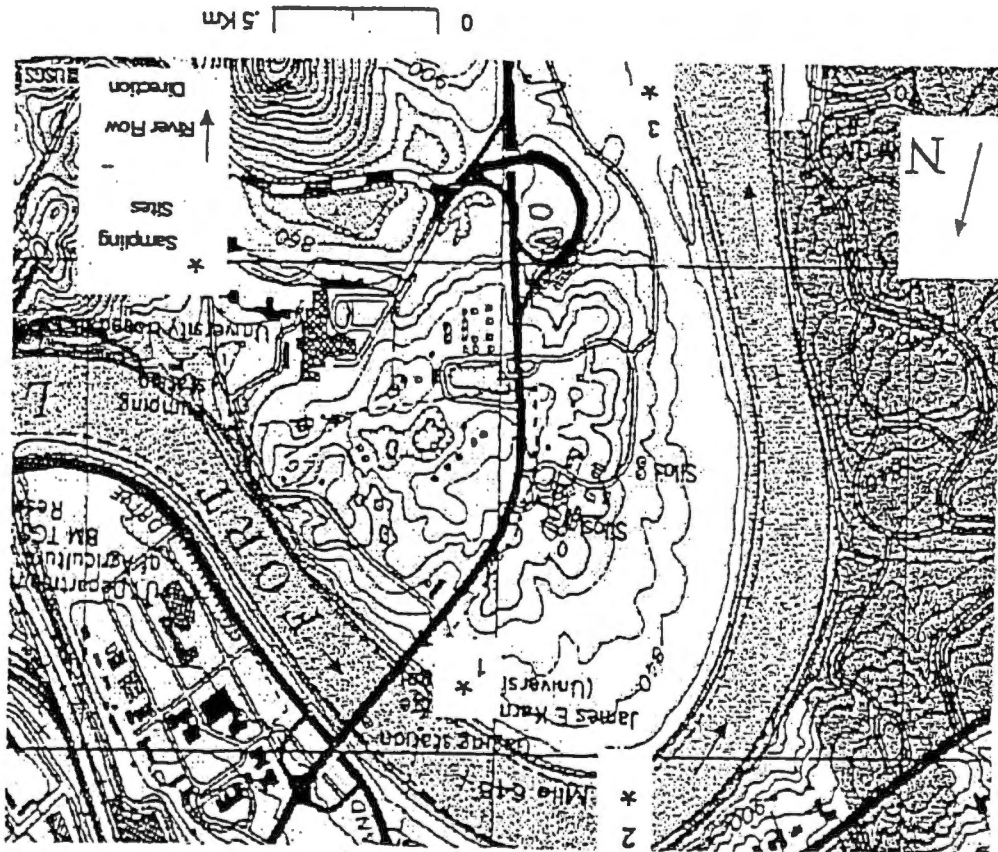
^b 0.7 km downstream from the baseline site

^c 2.08 km downstream from the baseline site

^d Not detected

^e "Listeria like" isolates: shared similar biochemical confirmation reactions with two species

Figure 1. Topographical Map of Tennessee River water sampling sites adjacent to the Dairy Farm



(Knoxville Experimental Station). *Listeria monocytogenes* was not isolated from the river water just before the beginning of the farm under a highway bridge. This point is referred to as the baseline because it is not influenced by potential farm run-off. The highest incidence of *L. monocytogenes* from the river was at the end of the farm (80%). *L. monocytogenes* was also isolated at a lower frequency from the river at the middle of the farm (20%). In each of the three river water sites sampled, *L. innocua* was detected in low, but constant percentages (Table 7). *L. grayi* and *L. ivanovii* was not isolated from the three river water sites. The greatest percentage of *Listeria*-like isolates (*Listeria* spp.) was found in the river water at the end of the farm (Table 7). This present study confirms the results obtained by Arumugaswamy and Gibson (1999) while working in New South Wales (Australia). These researchers found that 59% of the 27 samples they collected from the course of the Windsor (41% of 17 samples) and Georges Rivers (20% of the samples) tested positive for *Listeria* spp. Of the 27 samples, 33.3% were confirmed as *L. monocytogenes*. Arumugaswamy and Gibson (1999) suggested that the higher proportion of positive samples from the Windsor River was due to the number of outfalls from farmlands located along its banks.

The topography of the farm and its proximity to the Tennessee River may be seen in Figure 1. Three sites were sampled: river water before the farm (baseline data), river water at the middle of the farm (0.7 km), and river water below the farm (1.2 km). These data suggest a causal relationship between river contamination and farm effluent. *Listeria* in the river at the beginning of the farm may be partially due to the combination of the residential sewer overflow, improper disposal of waste from recreation boats, and waste matter from the birds that gather and nest under the highway bridge. The Knoxville City Wastewater treatment plant is approximately 1.2 km upstream from the farm. Effluent from this treatment facility is not likely to have an effect on the presence of *Listeria* since the effluent is normally chlorinated. Conversely, Combarro and associates (1997) while working in Spain, observed an increase in the levels of

Listeria spp. downstream from a sewage treatment plant. *L. monocytogenes* was isolated from the influent (58.8%), the effluent (58.1%), as well as the river water (44.3%) (Combarro et al., 1997). *L. seeligeri*, *L. welshimeri*, and *L. ivanovii* were also found in very low populations in the Spanish river samples (Combarro et al., 1997).

Listeria monocytogenes was not detected in the baseline river samples above the farm. The detection of *L. monocytogenes* at the middle sampling site shows the effects of farm run-off. This is further established by the fact that isolation percentage of *L. monocytogenes* isolates increase four-fold at the end of the river indicating that the river current is carrying contamination down-stream from the middle sampling site (Table 7). At the last sampling site, detection of *Listeria*-like isolates (*Listeria* spp.) showed an increase compared to the samples taken before the beginning of the farm (baseline) and from the middle sampling site (Table 7). This may be due to the accumulated water run-off from the farm. There was also a noticeable collection of organic material (leaves and sticks) along the banks of the river at the middle sampling site, which may facilitate the growth of *Listeria* in the waters at this location.

Optimization for recovery of *L. monocytogenes*

The secondary objective of this study was to determine the optimal recovery protocols for *L. monocytogenes* from the various types of samples collected. Figure 2 shows that MOX medium was similar to PALCAM medium for the isolation of *L. monocytogenes* from hair (8.1% and 6.1%), oral cavities (15.0% and 4.1%), and rectums (6.5% and 6.4%) of calves. PALCAM demonstrated a better recovery than MOX for the detection of *Listeria* spp. from hair (59.2% and 35.5%), oral cavities (64.3% and 37.5%), and rectums (54.2% and 43.5%) of calves (Figure 2). MOX medium was also superior to PALCAM medium for the isolation of *L. monocytogenes* and *Listeria* spp. from foremilk, hair, oral, and teat sites on cows. However, PALCAM was better than MOX for the isolation of *L. monocytogenes* and *Listeria* spp. from heavily contaminated samples such as from the rectums of cows due to its better selectivity (Figure 3). Sampling

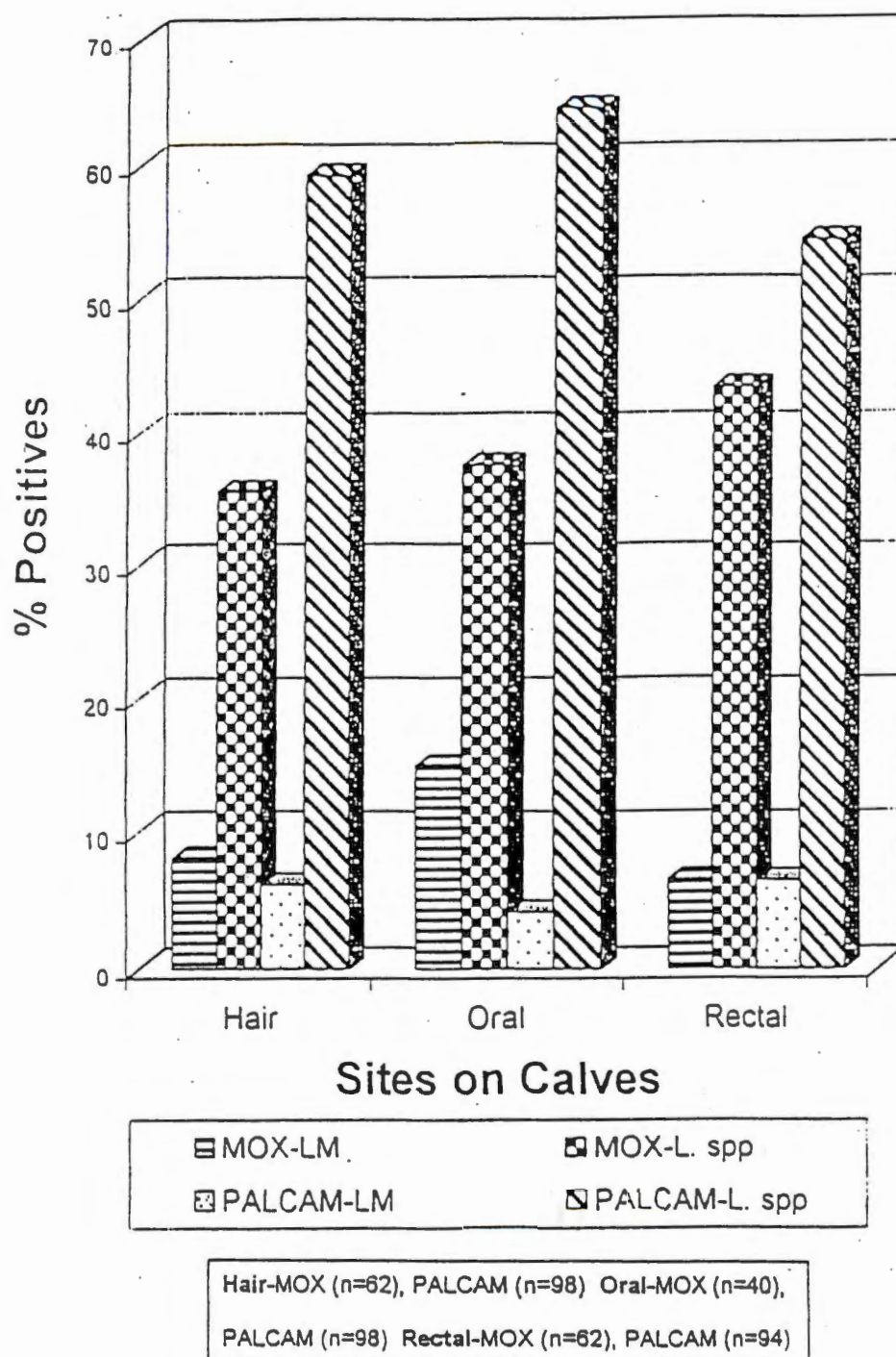


Figure 2. Comparison of MOX & PALCAM for the isolation of *L. monocytogenes* (LM) and *Listeria* spp. from Dairy Calves

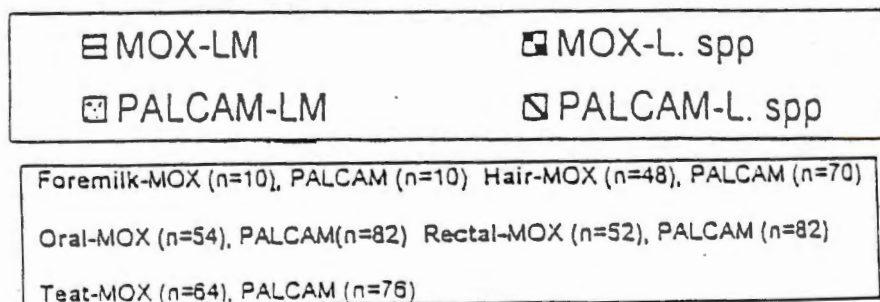
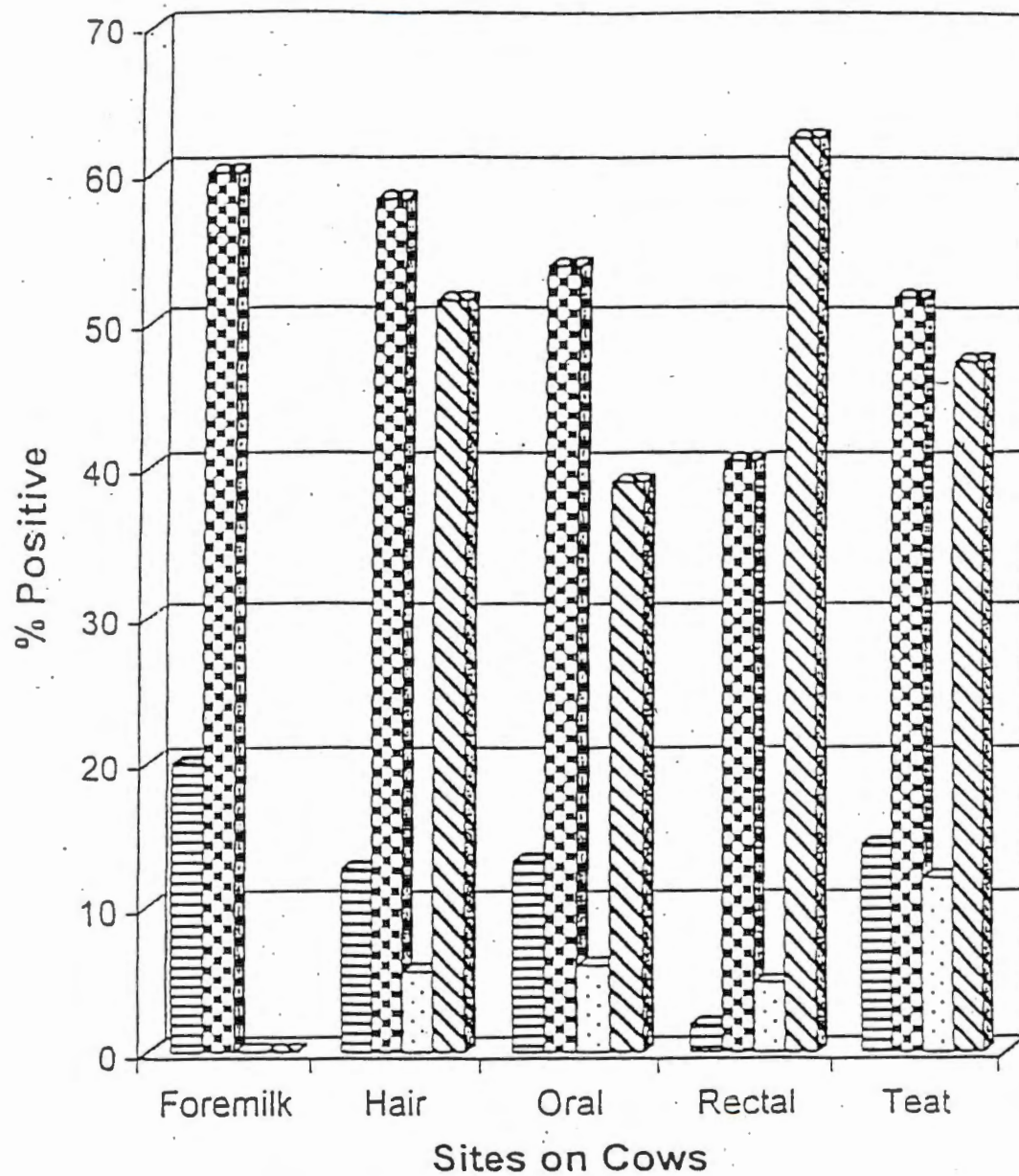


Figure 3. Comparison of MOX & PALCAM for the isolation of *L. monocytogenes* (LM) and *Listeria* spp. from Dairy Cows

locations for cows (anal, foremilk, hair, oral, and teat sites) and calves (anal, hair, and from the oral cavity) were not significantly different ($P>0.05$) for the isolation of *L. monocytogenes* although, *L. monocytogenes* and *L. seeligeri* were the only two species detected in the foremilk. Detection of other *Listeria* spp. from the foremilk on PALCAM medium was significantly different ($P<0.05$) than all other sampling locations (rectal, hair, oral, teat) on cows.

MOX medium showed a greater recovery than PALCAM medium for the isolation of *L. monocytogenes* from the bulk tank milk (28.1% and 15.6%), foremilk (20.0% and 0.0%), cups (11.8% and 0.0%), hose (5.6% and 3.6%), inflations (11.8% and 0.0%) (Figure 4). MOX medium was more suitable than PALCAM medium for the detection of other *Listeria* spp. from the foremilk, hose, and inflations. However, PALCAM was more efficient than MOX for the detection of *Listeria* spp. from the bulk tank milk and cups (Figure 4).

As illustrated on Figure 5, MOX medium was more suitable than PALCAM medium for the isolation of *L. monocytogenes* from the air (3.3% and 0.0%), bedding (13.8% and 5.6%), and insect (32.6% and 28.3%) samples. Isolation of *L. monocytogenes* on MOX was significantly greater ($P<0.05$) for insects compared to the other environmental sampling sites. Detection of *L. monocytogenes* on PALCAM medium was significantly different ($P<0.05$) for insect samples, but not for all other environmental sampling locations. PALCAM was preferable to MOX for the detection of *L. monocytogenes* from soil (6.6% and 2.6%). Figure 5 illustrates that PALCAM was preferable to MOX for the detection of *Listeria* spp. from air, bedding, and soil samples. MOX was more efficient than PALCAM for the isolation of *Listeria* spp. from insects (46.7% and 43.5%) (Figure 5).

MOX medium was better than PALCAM medium for the isolation of *L. monocytogenes* from feedbunk (18.8% and 10.9%), grain (13.5% and 4.8%), and silage (26.5% and 3.8%) (Figure 6). Detection of other *Listeria* spp. on MOX was significantly greater ($P<0.05$) from grain than from all other environmental sampling locations. Conversely, PALCAM had a greater recovery

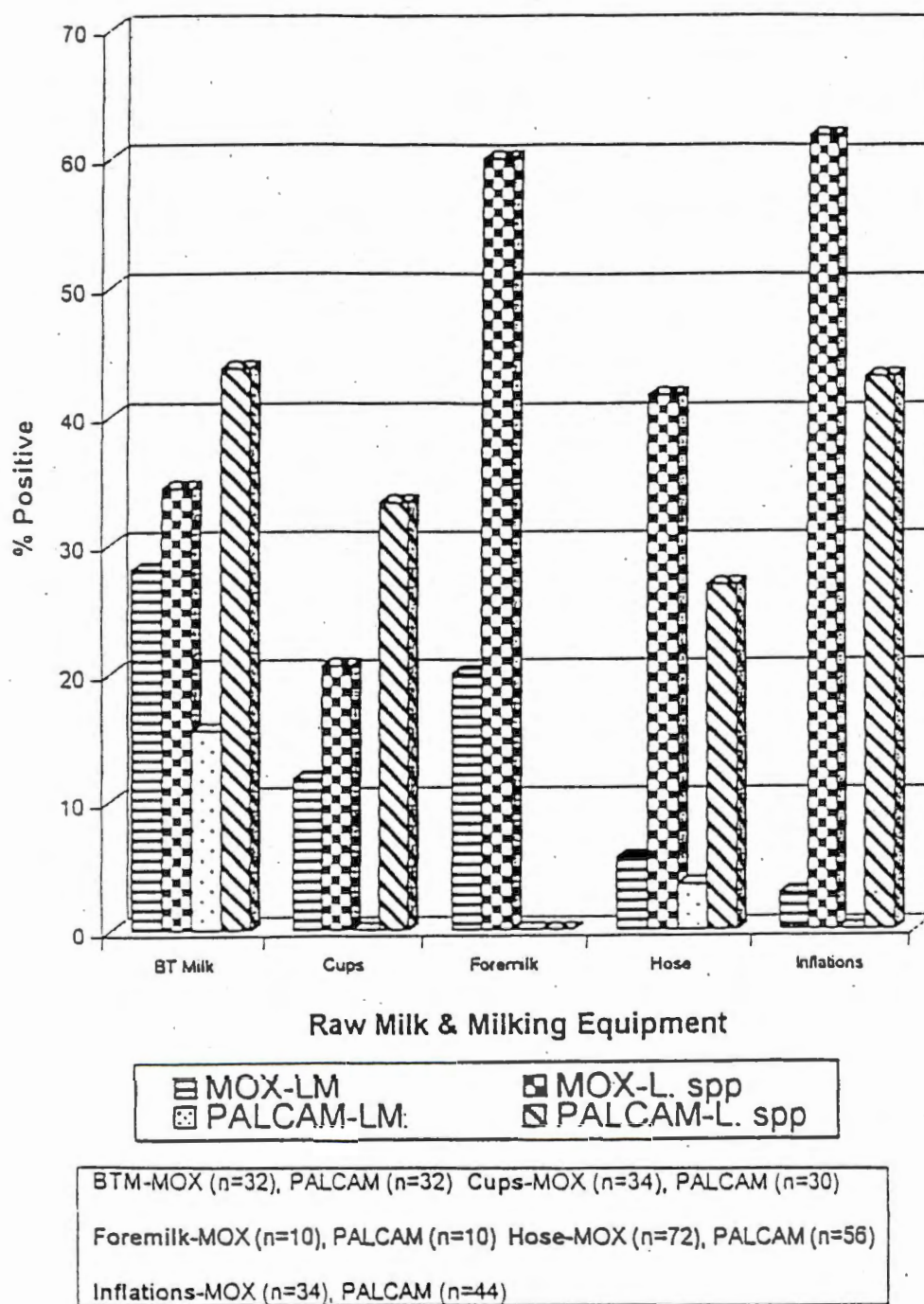


Figure 4. Comparison of MOX & PALCAM for the isolation of *L. monocytogenes* (LM) and *Listeria* spp. from Raw Milk and Milking Equipment

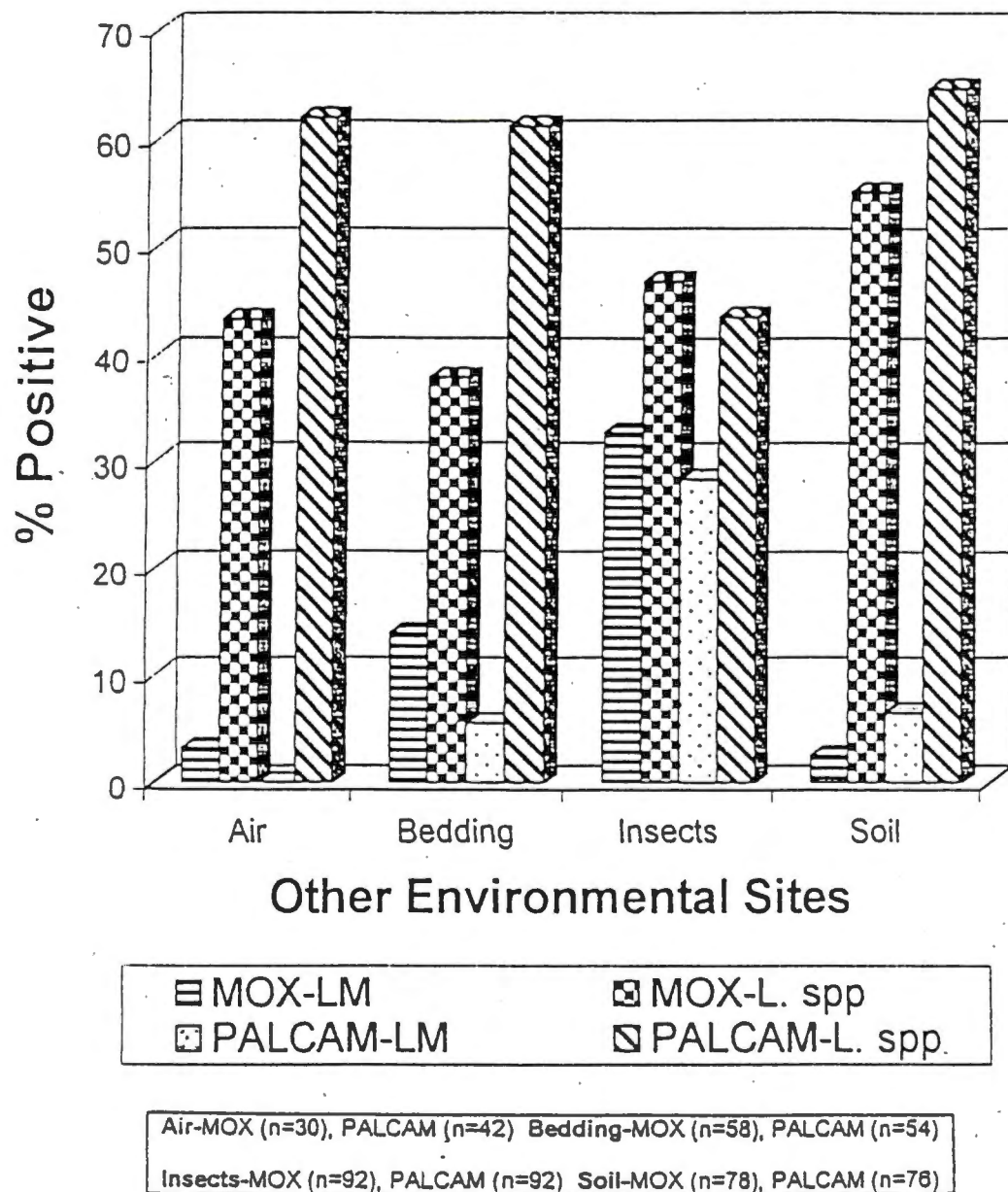


Figure 5. Comparison of MOX & PALCAM for the isolation of *L. monocytogenes* (LM) and *Listeria* spp. from other Environmental sites

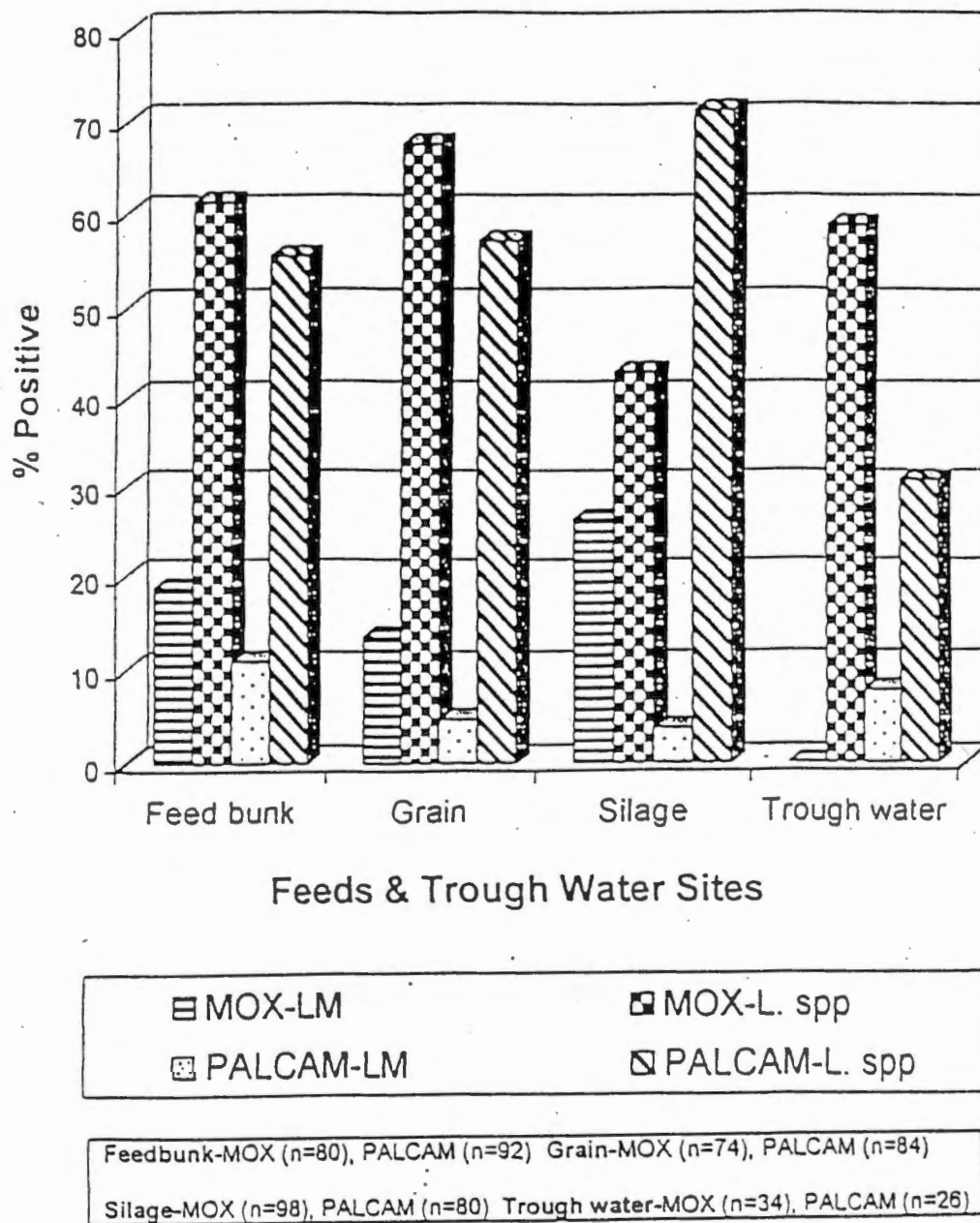


Figure 6. Comparison of MOX & PALCAM for the isolation of *L. monocytogenes* (LM) and *Listeria* spp. from Feeds and Trough water sites

over MOX for the detection of *L. monocytogenes* in trough water (7.7% and 0.0%) (Figure 6). PALCAM had a greater recovery over MOX for the isolation of *Listeria* spp. from silage (71.3% and 42.9%). MOX was more suitable than PALCAM for the detection of *Listeria* spp. from feedbunk (61.2% and 55.4%), grain (67.6% and 57.1%), and trough water (58.8% and 30.8%) (Figure 6).

MOX was better than PALCAM for the recovery of *L. monocytogenes* from river water at the middle (8.3% and 0.0%) and at the end (23.1% and 19.0%) of the farm (Figure 7). Neither medium isolated *L. monocytogenes* from the river water at the beginning of the farm (Figure 7). PALCAM showed an increased recovery over MOX for the isolation of *Listeria* spp. from river water at all three sites (bridge, middle, and end) along the farm (Figure 7).

Based on the enrichment and isolation protocols evaluated in this research, the authors recommend that MOX be used for all sample types associated with dairy farms with the exception of soil, rectums of cows, and trough water, in which PALCAM gives better isolation of *L. monocytogenes*. PALCAM was significantly better ($P<0.05$) for the selection of *Listeria* spp. other than *L. monocytogenes* from dairy farm environmental samples and thus gave a higher presumptive positive for *Listeria* spp. This increases the workload as well as the risk for false positives and false negatives. There was a significant difference ($P<0.05$) between the detection of no colonies, not *Listeria* isolates, *L. monocytogenes*, and other *Listeria* spp. on MOX and PALCAM for all sampling sites among the dairy farm environment. However, there was no significant difference ($P>0.05$) between the detection of no colonies, not *Listeria* isolates, *L. monocytogenes*, and other *Listeria* spp. on MOX and PALCAM for all sampling sites on cows and calves.

Sources of contamination by *Listeria* on the dairy farm

Ryser and Marth (1999) state that the majority of animal feeds, hay, and cereal grains have decreased levels of available water, thus restricting multiplication of *L. monocytogenes*.

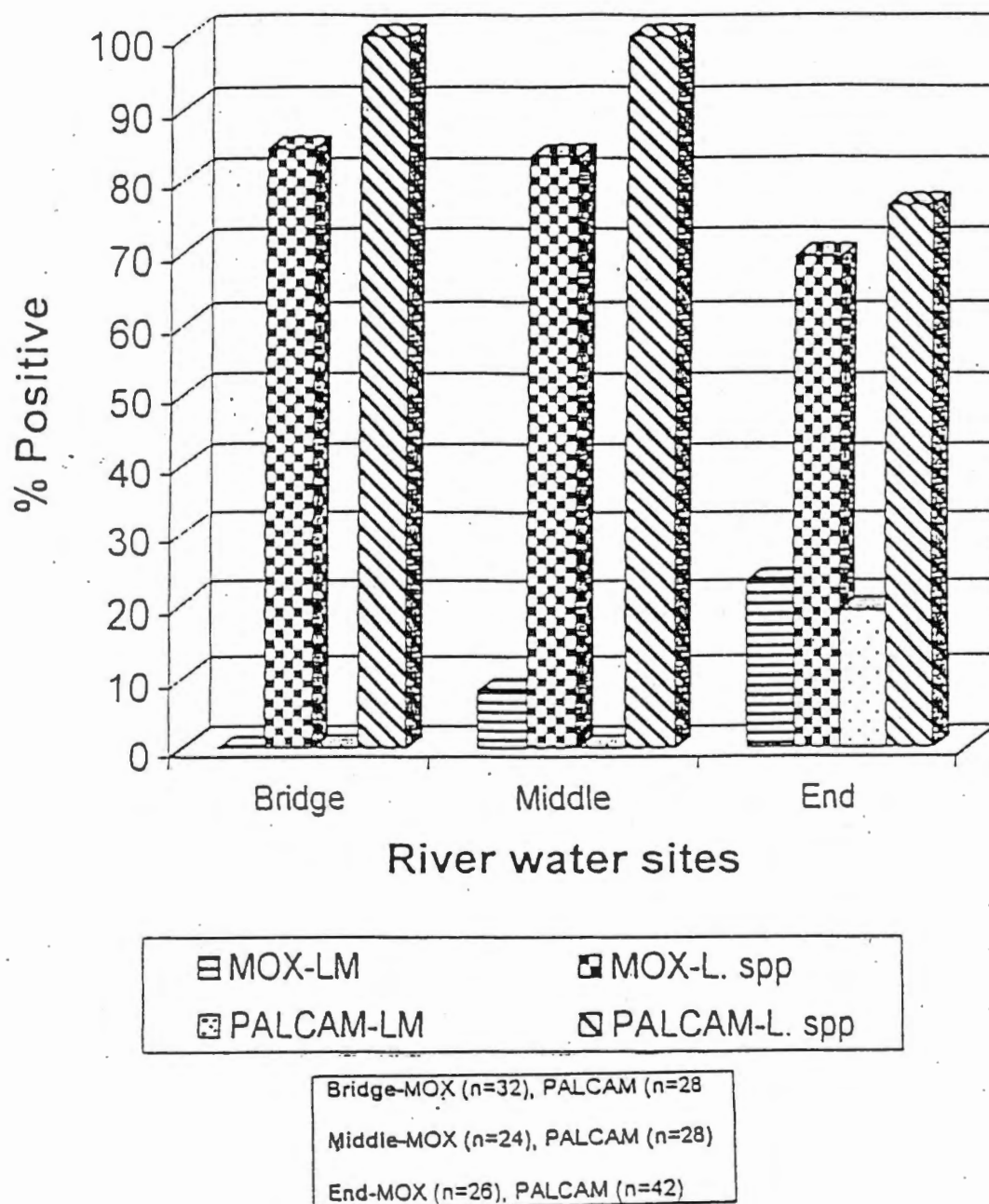


Figure 7. Comparison of MOX & PALCAM for the isolation of *L. monocytogenes* (LM) and *Listeria* spp. from Tennessee River water sites adjacent to the dairy farm

However, *L. monocytogenes* has been associated with these materials (Garcia et al., 1996), although the numbers are unlikely to reach levels that may cause a serious risk to farm animals. Formulated feeds in pellet form receive a heat treatment, which is capable of killing a large proportion, if not all, *Listeria* present (Ryser and Marth 1999). The incidence of *L. monocytogenes* in the oral sites of cows and calves both occurred at a level of 33% (Tables 5 and 6). This may be related to cows' exposure to grain, silage, and feedbunk, which serves as their primary food source. The calves were fed a diet of heat-treated feed pellets. Since many of these calves were not totally weaned or only recently weaned the teat of the cow may have accounted for the 33% incidence of *L. monocytogenes* in the oral cavities of calves. Rectal swabs revealed a higher incidence of *L. monocytogenes* in cows (20%) than in calves (13%), which may be attributed to the diets of cows versus calves. The grain, silage, and feedbunk fed to cows consistently tested positive for the presence of *L. monocytogenes* during this study. In comparison, calves were not exposed to this steady diet of grain, silage, and feedbunk. However, the comparison between heat-treated feed pellets fed to calves and silage, grain, and feedbunk fed to the cows was not performed during this research. The relationship between these various feeds on this farm should be investigated. Enumerating the amount of *Listeria monocytogenes* present on dairy cows, calves, and in the farm environment at different seasons of the year is also necessary to determine the levels of *L. monocytogenes* that cows and calves may be exposed to and when its occurrence in the environment is greatest.

The greatest isolation frequency of *L. monocytogenes* was found in the farm environment (Table 1). Thus the environment appears to be a constant source of contamination for dairy cows and calves. Dairy cows and calves had a higher isolation frequency of *Listeria* spp. than *L. monocytogenes*. This may be related to cow and calf mobility in and around the farm environment where the opportunity for exposure to various environmental *Listeria* exists. In addition, insects that frequent numerous sources of contamination on the farm may be a constant

source of *Listeria* for the farm animals. According to the data in Tables 2 and 6, there appears to be a direct pathway of *L. monocytogenes*, *L. seeligeri* and other *Listeria*-like (*Listeria* spp.) bacterial contamination beginning at the teats and foremilk traveling through the milking equipment (inflations, cups, hoses) and into the bulk tank. A causal factor of this infectious pathway may be the exposure of the teats to contaminated bedding, soil, fecal material, insects, and improperly sanitized milking equipment, which may transfer listeric infections to other cows at milking. Teats become contaminated via exposure to soil, bedding, and waste material when cattle lie down on bedding or soil. Previous studies have supported the link between on-farm sources of *Listeria* contamination (dairy cattle, raw milk, silage) and subsequent contamination of dairy processing environments, stress the importance of farm-based pathogens control program for listeriae (Arimi et al., 1997).

Dairy farmers need to follow a proactive approach in order to address on-farm food safety and animal health concerns for the dairy industry. It is imperative to minimize the continual influx of *Listeria monocytogenes* at the beginning of the food chain. Education in infection control practices for dairy farm managers and farm hands is an important first step in achieving this goal. A sound educational program would include information on cleaning and sanitizing farm equipment, feed passes and watering troughs, milking equipment (including the bulk tank), utilizing an approved teat dip, washing animal teats before milking, cleaning bedding areas in the absence of dairy cattle, the proper disposal of animal waste, hand washing for farm workers, the avoidance of spreading manure on farm fields, the importance of culling infected or mastitic cattle out of the herd, the elimination of wood chips for bedding material, the removal of feeding and watering troughs made of wood, and controlling access of animals to rivers and streams.

In addition to initiating a sound educational program, a cost effective, comprehensive sampling plan, utilizing optimal enrichment and plating media for the recovery of *Listeria* spp.,

particularly *L. monocytogenes*, and identification of reservoirs specific to a site, should be initiated. Dairy processors in contractual agreement with the dairy farmers would provide the funding necessary to ensure a broad, diverse, and effective education and sampling program. Oversight of this program should be conducted by food safety and animal health regulatory agencies. This approach would be useful in the reduction of *Listeria* from dairy farm sites and will provide a continual surveillance for contamination on the farm to ensure quality control and sound risk management decisions.

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PART III

**GENOTYPIC DIVERSITY OF *LISTERIA* ISOLATES RECOVERED FROM DAIRY
COWS, CALVES, AND THE FARM ENVIRONMENT**

ABSTRACT

Listeria isolates (n=48) from a total of 548 cow, 454 calf, and 1,556 University of Tennessee Dairy Farm environmental isolates obtained during a 1999-2001 survey were submitted for automated ribotype analysis utilizing the Riboprinter microbial characterization system, alpha version (E. I. Du Pont de Nemours & Co., Inc.). This investigation compared *Listeria* ribotypes (RTs) obtained from dairy cows, calves, and farm environmental isolates to a database of clinical isolates of *Listeria* associated with animal miscarriages and foodborne listeriosis. Riboprint patterns of 17 of the 48 isolates confirmed the identity as *Listeria* spp. based upon the Du Pont (*EcoRI*) database. Fourteen *Listeria* RTs were discriminated. The 14 RTs were identified as 5 *L. monocytogenes* RTs (1029, 1041, 1051, 1052, and 1XXXX, a confidential isolate held by Du Pont), 5 *L. innocua* RTs (1005, 1006, 1009, 1010, and 1019), and 4 *L. welshimeri* RTs (1072, 1073, 1074, and 1079). Of the isolates selected for ribotyping, *L. monocytogenes* was detected phenotypically from grain, feedbunk, corn silage, raw bulk tank milk, air, soil, bedding, river water adjacent to the midpoint of the farm, milking equipment, calf oral and hair samples, as well as cow anal, oral, and teat samples. According to ribotype analysis, optimal detection sites for the presence of *L. monocytogenes* were from the cows' teats, the oral cavity of calves, corn silage, raw bulk tank milk, and from the river water adjacent to the midpoint of the farm. Knowledge of the agricultural ecosystem, the taxonomy of *Listeria* strains, and careful attention to detailed isolation and confirmation protocols were essential to prevent misidentification of *Listeria* spp. when initiating and conducting an on-farm pathogen-reduction program.

INTRODUCTION

Repeated outbreaks of foodborne listeriosis in the 1980s have increased the interest in the epidemiology of this disease (Boerlin and Piffaretti, 1991). The use of advanced molecular fingerprinting techniques have aided in identifying sources and strains of *Listeria monocytogenes* as well as to narrow the scope of epidemiological investigations. The application of serotyping, phage typing, and restriction fragment length polymorphism analysis in combination have facilitated the detection of persistent strains of *L. monocytogenes* for up to 9 months in bulk tank milk (Harvey and Gilmour, 1994). A broad range of *Listeria* electrophoretic types are present at the primary production environment. Virulent strains arise at the farm level and occasionally contaminate raw food material. These strains may also adapt to the food-processing environment. This occurrence may lead to widespread contamination of food products with *L. monocytogenes* (Fenlon et al., 1996). Molecular typing methods that have been previously used to successfully trace the transmission of human listeriosis back to foods of animal origin (meat and dairy products), verify epidemiological associations between silage consumption, cattle and sheep listeriosis, shedding of *L. monocytogenes* in raw milk, and distinguish a select number of *Listeria* isolates from food processing environments (Batt, 1997). These techniques include restriction enzyme analysis (Wesley and Ashton, 1991), multilocus enzyme electrophoresis (Boerlin and Piffaretti, 1991; Graves et al., 1994), randomly amplified polymorphic DNA (Czajka and Batt, 1994; Lawrence et al., 1993; Farber and Addison, 1994), pulsed-field gel electrophoresis (Louie et al., 1996), arbitrarily primed PCR (Louie et al., 1996), and non-automated ribotyping (Louie et al., 1996; Graves et al., 1994; Swaminathan et al., 1996).

Due to the ubiquitous nature of *L. monocytogenes* in the environment a rapid, highly discriminatory typing system is needed to differentiate bacterial isolates beyond the species and subspecies level. Recently, studies have reported the applicability of automated ribotyping, which employs the use of a database of ribotype patterns with a common nomenclature to

determine the significance of given ribotypes for isolates obtained from listeriosis outbreaks (Wiedmann et al., 1996; Ryser et al., 1997). Consistent detection and identification of *Listeria* spp., particularly *L. monocytogenes* from dairy cattle and the farm environment, is essential to ensure valid data for risk management decisions.

Several of the genomic typing applications, which include non-automated ribotyping, are time consuming, laborious, and sometimes produce data that are unclear (Arimi et al., 1997). An automated ribotyping technique (Qualicon Riboprinter Microbial Characterization System, alpha version, E. I. Du Pont de Nemours and Co. Inc., Wilmington, DE) (Bruce et al., 1995; Hubner et al., 1995) is available which will identify selected *Listeria* strains and other bacterial pathogens to the species level. The riboprinter can also provide discrimination beyond the species level, and characterize strains via a six-stage automated process. This automated system provides typing data, which are normalized, and allows the data to be compared between various laboratories. A database of ribotype patterns with a common nomenclature can be utilized to determine the significance of a given ribotype in clinical and epidemiologically implicated feed or environmental samples (Wiedmann et al., 1997). Other studies have demonstrated the use of automated ribotype analysis to type *Listeria* isolates obtained from silage (Ryser et al., 1997; Arimi et al., 1997; Wiedmann et al., 1996), to investigate potential sources of an epizootic listerial encephalitis in ruminants (Wiedmann et al., 1996; Wiedmann et al., 1997), and to evaluate *Listeria* contaminated raw refrigerated meat and poultry products (Ryser et al., 1996). Automated ribotyping has also been used to examine the geographic strain distribution of *L. monocytogenes* isolated from smoked salmon (Gendel and Ulaszek, 2000) and to assess the incidence and diversity of *Listeria* isolates from the dairy processing environment, raw bulk tank milk, and from udder quarter milk (Arimi et al., 1997).

Epidemiological data on *Listeria* contamination are needed to develop on-farm pathogen-control programs (Cullor, 1995). Such data are essential for risk assessment and risk

management decisions. Therefore, in this investigation, automated ribotype analysis was utilized to discriminate *Listeria* isolates obtained from a survey of cows, calves, and farm environmental sites at the University of Tennessee Dairy Farm. In addition, the *Listeria* ribotypes obtained from dairy cattle and farm environmental isolates were compared with a database of clinical isolates of *Listeria*, which have previously been associated with both animal miscarriages and human cases linked to outbreaks of foodborne listeriosis.

MATERIALS AND METHODS

Selection and preparation of *Listeria* isolates

A total of 48 *Listeria* isolates were selected to represent as many phenotypic variations and sample sites as possible from 548 cow samples (hair, oral, teat, anal, and foremilk samples), 454 calf samples (hair, oral, and anal), and 1,556 farm environmental samples [air, bedding, bulk tank milk, feedbunk, grain, milking equipment (inflatons, cups, hoses), soil, silage, insects, farmyard soil, river water, and trough water]. The isolates were collected as part of a survey of the University of Tennessee Dairy Farm (Knoxville Experimental Station) during the months of November 1999 through May 2001 and were confirmed for the presence or absence of *Listeria* species using the typical differential biochemical reactions illustrated in Table 1. These isolates were stored at -70°C in 20% skim milk (Kroger, Knoxville, TN) supplemented with 20% glycerol (Sigma Chemical Co., St. Louis, MO). Prior to ribotyping, each isolate was removed from -70°C storage, streaked for reisolation onto brain heart infusion agar (BHIA) (Difco Laboratories, Detroit, MI), biochemically reconfirmed, and subcultured twice to obtain a lawn growth on brain heart infusion agar (BHIA) (Difco) (35°C for 18 to 24 hours). Using a colony pick, two colonies were taken from each BHIA (Difco) plate and resuspended in 40µl of buffer (20mM Tris and 20mM EDTA, pH=7.5) in a microcentrifuge tube. The remainder of the analysis was performed as described by the manufacturer of the instrument and briefly involved: transferring eight different diluted samples (30µl) into separate wells of a sample carrier, transferring the sample

Table 1. Differential characteristics of the species of the genus *Listeria*^{a,b,c}

Characteristics	<i>L.</i> <i>grayi</i>	<i>L.</i> <i>innocua</i>	<i>L.</i> <i>ivanovii</i>	<i>L.</i> <i>monocytogenes</i>	<i>L.</i> <i>seeligeri</i>	<i>L.</i> <i>welshimeri</i>
β-Hemolysis	-	-	+ ^a	+ ^f	+	-
CAMP test (<i>Staphylococcus aureus</i>)	-	-	-	+	+	-
Catalase production	+	+	+	+	+	+
Acid production from:						
Mannitol	+	-	-	-	-	-
L-Rhamnose	-	+/- ^d	-	+	-	+/-
D-Xylose	-	-	+	-	+	+
^a Symbols: +, 90% or more of strains are positive; -, 90% or more of strains are negative; (+/-), 11- 89% of strains are positive (1994). ^b The species <i>Listeria denitrificans</i> has been transferred to a separate genus <i>Jonesia</i> in Group 20 in the Bergey's Manual of Systematic Bacteriology. ^c All species within the genus <i>Listeria</i> are Gram-positive rods and motile (usually characteristic "umbrella motility") at 22-25°C, however motility does not typically occur at 37°C. ^d Partial fermentation of the sugar ^e Usually a wide zone or multiple zones ^f A few strains negative						

As adopted and modified from (Holt et al., 1994).

carrier to a heating block at 80°C for 10 minutes to kill the cells and inactivate nucleases, adding lytic enzymes to each sample well, loading the carrier, probe, substrate, conjugate, MP base, DNA prep carrier with the *EcoRI* enzyme, nylon membrane, gel cassette, and purified water onto the instrument and closing the front glass doors to initiate ribotyping.

Ribotype analysis

Ribotype analysis was used to identify the selected *Listeria* isolates to the species level, and to provide discrimination beyond the species level. The procedure involved lysing *Listeria* cells, performing a restriction enzyme (*EcoRI*) digestion of the chromosomal DNA, separating the restriction fragments by agarose gel electrophoresis, transferring fragments to a nylon membrane, performing a Southern Blot hybridization via a chemiluminescent ribosomal probe (*E. coli rrnB* rRNA operon) of a nylon membrane, photographing the luminescent pattern image from the agarose gel using a charged-coupled device (CCD) camera, and provided data processing of the digital image. A DNA fragment pattern based on fragment size was produced based upon generation of rRNA restriction fragment length polymorphisms (RFLP) using a probe that hybridizes to an array of rRNA operons located on various restriction fragments. Riboprint patterns were identified, compared to a library of patterns in Du Pont database, and classified according to the software of the Qualicon riboprinter system (Hubner, 1989). To evaluate the performance of the riboprinter a set of control strains consisting of *Escherichia coli*, *Staphylococcus aureus*, *Salmonella enterica* Serovar Infantis, and *Listeria innocua* were ribotyped in duplicate after the completion of five batches (40 RTs) of presumptive Tennessee Dairy Farm *Listeria* isolates.

RESULTS AND DISCUSSION

Diversity of *Listeria* RTs among the dairy farm

Biochemically confirmed *Listeria* isolates (n=48) were obtained from a previous survey from November 1999 through May 2001 of dairy cows, calves, and various University of

Tennessee Dairy Farm (Knoxville Experimental Station) environmental sites. Presumptive *Listeria* isolates were re-evaluated using strain-specific automated ribotype analysis. Riboprint patterns of 17 of the 48 isolates confirmed the identity as *Listeria* spp. based upon the Du Pont (*EcoRI*) database. The 17 *Listeria* isolates were discriminated as 5 *L. monocytogenes* RTs (1029, 1041, 1051, 1052, and 1XXXX, a confidential isolate held by Du Pont), 5 *L. innocua* RTs (1005, 1006, 1009, 1010, and 1019), and 4 *L. welshimeri* RTs (1072, 1073, 1074, and 1079). *Listeria* ribotypes of the University of Tennessee Dairy Farm (Knoxville Experimental Station) isolate sources are identified on Table 2.

Cows

Cow teat RTs showed the presence of *L. monocytogenes* RTs (1029, 1041, and 1051) and *L. innocua* RTs (1006 and 1010) (Table 2). This finding indicates the possible presence of multiple *Listeria* species and strains isolated from the same cow teat sample. *L. monocytogenes* was detected using MOX medium, while the *L. innocua* was isolated using PALCAM medium. Four different cow teat samples, which ribotyped as *L. monocytogenes* RT (1041, 1051, and two as 1029) (Table 2) had the exact same phenotypic reactions and were all confirmed as *L. monocytogenes* because they had reactions typical for *L. monocytogenes* according to Table 1. The last cow teat isolate, which was typed as *L. innocua* RT (1010) was conventionally identified as *L. seeligeri* because of its positive xylose test even though there was also acid production from rhamnose. Also, of importance, this colony showed umbrella motility at both 25°C and 37°C. The usual reactions for *L. seeligeri* as well as *L. innocua* are shown in Table 1. Among rectal, hair, and oral cow isolates, no *Listeria* RTs were found. Among two cow rectal isolates biochemically identified as *L. monocytogenes* and *L. seeligeri* were genotypically found to be *Lactobacillus sanfranciscensis* RT (13483) and *Staphylococcus xylosus* RT (4243), respectively (Table 3). The suspect *L. monocytogenes* colony was motile at 25°C, but not motile at 37°C, was

Table 2. A summary of biochemical confirmations and ribotypes (RTs) of *Listeria* isolates obtained from Cows, Calves, and the University of Tennessee (UT) Dairy Farm.

DuPont Nearest Isolation ID ^a	DuPont Riboprimer bacterial identification	Gram Stain	Catalase	Mannitol	Rhamnose	Xylose	Motility ^d at 35°C	Motility ^d at 37°C	β -hemo ^f	CAMP ^g	Isolation Media ⁱ	Phenotypic Confirmation <i>Listeria</i> species	UT Dairy Farm Sources
1005	<i>L. innocua</i>	+	+	-	+/-	-	UM ^h	UM	-	-	PAL	<i>L. innocua</i>	Bulk Tank Milk
1006	<i>L. innocua</i>	+	+	-	+	-	UM	UM	-	+	MOX	<i>L. monocytogenes</i>	Grain
1006	<i>L. innocua</i>	+	+	-	+	-	UM	UM	+	-	MOX	<i>L. innocua</i>	Insects
1006	<i>L. innocua</i>	+	+	-	+	-	UM	UM	+	-	MOX	<i>L. innocua</i>	Insects
1009	<i>L. innocua</i>	+	+	-	+/-	-	UM	UM	+	+	MOX	<i>L. monocytogenes</i>	Feedbunk ^k
1010	<i>L. innocua</i>	+	+	-	+	-	UM	UM	-	+	MOX	<i>L. monocytogenes</i>	Oral calf
1010	<i>L. innocua</i>	+	+	-	+	+	UM	UM	+	+	PAL	<i>L. seeligeri</i>	Cow teat
1019	<i>L. innocua</i>	+	+	-	+	-	UM	UM	+	+	MOX	<i>L. monocytogenes</i>	Bulk Tank Milk
1029	<i>L. monocytogenes</i>	+	+	-	+	-	UM	M	+	+	MOX	<i>L. monocytogenes</i>	Cow teat
1029	<i>L. monocytogenes</i>	+	+	-	+	-	UM	M	+	+	MOX	<i>L. monocytogenes</i>	Cow teat
1041	<i>L. monocytogenes</i>	+	+	-	+	-	UM	UM	-	+	MOX	<i>L. monocytogenes</i>	Oral calf
1041	<i>L. monocytogenes</i>	+	+	-	+	-	UM	M	+	+	MOX	<i>L. monocytogenes</i>	Cow teat
1051	<i>L. monocytogenes</i>	+	+	-	+	-	UM	M	+	+	MOX	<i>L. monocytogenes</i>	Cow teat
1052	<i>L. monocytogenes</i>	+	+	-	+	+	M ^l	M	+	+	MOX	<i>L. spp.</i> ^j	River End ^l
1XXXX ^b	<i>L. monocytogenes</i>	+	+	-	+	+	UM	UM	+	+	MOX	<i>L. monocytogenes</i>	Silage
1XXXX ^b	<i>L. monocytogenes</i>	+	+	-	+	-	UM	UM	+	+	MOX	<i>L. monocytogenes</i>	Silage
1072	<i>L. welshimeri</i>	+	+	-	-	+	UM	M	+	-	MOX	<i>L. welshimeri</i>	River Baseline ^m
1073	<i>L. welshimeri</i>	+	+	-	+	-	UM	M	+	+	PAL	<i>L. monocytogenes</i>	Feedbunk
1074	<i>L. welshimeri</i>	+	+	-	+/-	+	UM	M	-	+	MOX	<i>L. monocytogenes</i>	River Middle
1079	<i>L. welshimeri</i>	+	+	-	+/-	+	UM	M	-	+	MOX	<i>L. monocytogenes</i>	River Middle

^a Identification number^b Confidential isolate held by DuPont Qualicon^c Partial fermentation of the sugar^d Determined presence of motility at 48h and 7 days using motility medium S (Difco Laboratories, Detroit, MI) with addition of 1% - 2,3,5, triphenyltetrazolium chloride (Sigma Chemical Co., St. Louis, MO)^e Classic umbrella motility^f Motility: fanning out from the stab line in the culture medium^g β -hemolysis: performed via a stab inoculum of 5% sheep blood agar plates, incubating the plates at 35°C, and checking for hemolysis of blood cells at 24h and 48h^h Christie Atkins Munch-Peterson test using 5% sheep blood agar plates performed in the presence of *Staphylococcus aureus* ATCC 49444 (American Type Culture Collection, Rockville, MD), incubated plates at 35°C, and analyzed at 24h and 48hⁱ Modified Oxford agar (Difco Laboratories, Detroit, MI) or PALCAM agar (Difco Laboratories, Detroit, MI)^j *L. spp.* "Listeria-like" isolates, which shared similar biochemical confirmation reactions with two species^k A mixture of hay, silage, and grain^l Sampling site at the end of the farm 2.08 km downstream from the baseline site^m River water sampling site immediately before the beginning of the farm property

Table 3. A summary of the phenotypic characterization of "Listeria-like" isolates from Cows, Calves, and the University of Tennessee (UT) Dairy Farm and their genotypic identification according to the Du Pont Qualicon Riboprinter Microbial Characterization System Database®

Du Pont Nearest Neighbor ID ^a	Du Pont Riboprint Nearest neighbor bacterial Identification	Gram	Catalase	Mannitol	Rhamnose	Xylose	Motility ^b at 25°C	Motility ^b at 37°C	β -hemol	CAMP ^d	Isolation Media ^e	Phenotypic Confirmation Listeria species	UT Dairy Farm Sources
6233	<i>Enterococcus mundtii</i>	+	+	+	+	+	UM ^f	UM	+	+	MOX	<i>L. spp.</i> ^g	River Baseline ^h
12364	<i>Bacteroides thetaiotaomicron</i>	-	+	-	-	-	M ⁱ	NH ⁱ	-	-	MOX	<i>L. innocua</i>	River End ^h
6212	<i>Enterococcus faecalis</i>	+	+	+	-	+	M	M	+	+	PAL	<i>L. seeligeri</i>	Bulk Tank Milk
13103	<i>Enterococcus faecalis</i>	+	+	+	+	+	M	M	+	+	MOX	<i>L. monocytogenes</i>	Cups ^j
6203	<i>Enterococcus casseliflavus</i>	+	+	+	-	+	M	UM	-	+	PAL	<i>L. garsi</i>	Cups
6212	<i>Enterococcus faecalis</i>	+	+	-	+	+	M	M	+	+	PAL	<i>L. monocytogenes</i>	Inflations ^k
9300	<i>Bacillus subtilis</i>	+	+	+	-	+	M	M	+	+	PAL	<i>L. seeligeri</i>	Feedbunk ^l
14809	<i>Bacillus pasteurii</i>	+	+	-	-	+	UM	UM	+	+	MOX	<i>L. seeligeri</i>	Grain
6016	<i>Bacillus thuringiensis</i>	+	+	+	-	+	M	UM	+	+	MOX	<i>L. spp.</i>	Trough water
13146	<i>Acinetobacter baumannii</i>	-	+	-	+	+	-	M	+	+	MOX	<i>L. monocytogenes</i>	Bedding
1199	<i>Staphylococcus sciuri</i>	+	+	+	+	+	UM	M	+	+	PAL	<i>L. monocytogenes</i>	Bedding
11031	<i>Bacillus sphaericus</i>	+	+	-	+	+	UM	M	+	+	PAL	<i>L. monocytogenes</i>	Soil
12533	<i>Bacillus clausii</i>	+	+	-	-	-	UM	M	+	+	MOX	<i>L. spp.</i>	Soil
1009	<i>Escherichia coli</i>	-	+	-	-	+	M	M	-	+	MOX	<i>L. spp.</i>	Soil
6096 & 14766	<i>Bacillus sphaericus</i>	+	+	-	-	-	M	UM	+	+	MOX	<i>L. seeligeri</i>	Soil
10131	<i>Pseudomonas aeruginosa</i>	+	+	-	-	-	M	M	+	+	MOX	<i>L. seeligeri</i>	Air
13093	<i>Lactobacillus ruminis</i>	+	+	+	+	-	M	M	+	+	MOX	<i>L. monocytogenes</i>	Air
13638	<i>Bacillus luumolactis</i>	+	+	+	+	-	M	M	+	+	MOX	<i>L. monocytogenes</i>	Air
1207	<i>Staphylococcus sciuri</i>	+	+	+	-	+	M	M	+	+	PAL	<i>L. seeligeri</i>	Anal calf
1199	<i>Staphylococcus sciuri</i>	+	+	-	+	-	M	M	-	+	PAL	<i>L. monocytogenes</i>	Hair calf
6210	<i>Enterococcus faecalis</i>	+	+	-	-	-	M	M	-	-	MOX	<i>L. innocua</i>	Hair calf
13066	<i>Lactobacillus lactis</i>	+	+	-	-	-	M	M	+	+	MOX	<i>L. seeligeri</i>	Oral calf
4104	<i>Staphylococcus aureus</i>	+	+	+	+	+	M	M	+	+	PAL	<i>L. spp.</i>	Oral calf
13183	<i>Lactobacillus sanfranciscensis</i>	+	+	-	+	+	M	NH	-	+	PAL	<i>L. monocytogenes</i>	Rectal cow
4243	<i>Staphylococcus aureus</i>	+	+	+	-	+	M	M	+	+	PAL	<i>L. seeligeri</i>	Rectal cow
14136	<i>Enterobacter cloacae</i>	-	+	-	-	-	UM	UM	-	-	MOX	<i>L. innocua</i>	Hair cow
13030	<i>Bacteroides vulgatus</i>	-	+	-	+	+	M	M	-	+	PAL	<i>L. innocua</i>	Hair cow
15375 & 15123	<i>Klebsiella pneumoniae</i>	-	+	-	-	-	M	M	+	+	PAL	<i>L. innocua</i>	Hair cow
4105 & 4106	<i>Staphylococcus aureus</i>	+	+	+	-	-	M	M	+	+	PAL	<i>L. seeligeri</i>	Oral cow
6233	<i>Enterococcus mundtii</i>	+	+	-	+	+	M	M	+	+	MOX	<i>L. monocytogenes</i>	Oral cow
13479	<i>Lactobacillus reuteri</i>	+	+	-	-	-	M	M	+	+	MOX	<i>L. seeligeri</i>	Test cow

^a Identification number

^b Determined presence of motility at 48h and 7 days using motility medium 5 (Difco Laboratories, Detroit, MI) with addition of 1% - 2,3,5, triphenyltetrazolium chloride (Sigma Chemical Co., St. Louis, MO)

^c β -hemolysis: performed via a stab inoculum of 5% sheep blood agar plates, incubating the plates at 35°C, and checking for hemolysis of blood cells at 24h and 48h.

^d Christie Atkins Munch-Peterson test using 5% sheep blood agar plates performed in the presence of *Staphylococcus aureus* ATCC 49444 (American Type Culture Collection, Rockville, MD), incubated plates at 35°C, and analyzed at 24h and 48h

^e modified Oxford agar (Difco Laboratories, Detroit, MI) or PALCAM agar (Difco Laboratories, Detroit, MI)

^f Classic umbrella motility

^g *L. spp.* "Listeria-like" isolates, which shared similar biochemical confirmation reactions with two species

^h River water sampling site immediately before the beginning of the farm property

ⁱ Motility: fanning out from the stab line in the culture medium

^j Not motile

^k Sampling site at the end of the farm 2.08 km downstream from the baseline site

^l Partial fermentation of the sugar

^m Cups: central area where milk is sucked into from the inflations

ⁿ Inflations: apparatus attaching to each of the teats of lactating cows

^o A mixture of hay, silage, and grain

CAMP (Christie Atkins Munch-Peterson), xylose, and catalase positive, mannitol and β -hemolysis negative, and had a partial (+/-) rhamnose fermentation. These reactions deviated from the usual reaction for *L. monocytogenes* (Table 2) by only the presence of acid from xylose. The suspect *L. seeligeri* colony differed from the typical *L. seeligeri* reaction (Table 1) by only the presence of acid from mannitol. Three cow hair isolates all conventionally showed the presence of *L. innocua*. After Gram-stain analysis followed by ribotyping and identification via the nearest neighbor command, the isolates were found to be *Enterobacter cloacae* RT (14136), *Bacteroides vulgatus* RT (15030), and *Klebsiella pneumoniae* RTs (15375 and 15123) (Table 3). Among two cow oral isolates biochemically identified as *L. seeligeri* and *L. monocytogenes* were genotypically found via the nearest neighbor identification to be *Staphylococcus cohnii* RT (4105 and 4106) and *Enterococcus mundtii* RT (6235), respectively (Table 3). Both isolates were motile at 25°C and 37°C and were positive for the β -hemolysis and CAMP tests. The *L. seeligeri* colony showed a +/- for mannitol, and negative rhamnose and xylose. The presumptive *L. monocytogenes* isolate was the same as the usual reaction for *L. monocytogenes*, but the rhamnose revealed a partial sugar fermentation. Lastly, one teat isolate confirmed as *L. seeligeri*, showed a typical reaction for this species, however it ribotyped as *Lactobacillus reuteri* RT (13070) (Table 3). Teats may become contaminated via exposure to soil, bedding, and waste material when cattle lie down on bedding or soil.

Calves

Ribotype analysis of four calf oral colonies revealed the presence of *L. innocua* RT (1010), *L. monocytogenes* RT (1041), *Lactobacillus kefirgranum* RT (13066), and *Staphylococcus cohnii* RT (4104). These *Listeria* RTs (Table 2) were also isolated from a cow's teat sample which ribotyped as *L. monocytogenes* RT (1041) and *L. innocua* RT (1010), thus indicating that the calf may have been recently weaned at the time that both the cow and calf samples were taken. It is unknown if the calf sampled was recently weaned off of the teats of the

same cow that was sampled. *L. monocytogenes* RT (1041) was also isolated from a calf's rectal sample. This RT was phenotypically identified as *L. monocytogenes* because it demonstrated a typical reaction for *L. monocytogenes* (Table 2). The two non-Listeric RTs, *Lactobacillus kefirgranum* and *Staphylococcus cohnii* were conventionally confirmed as *L. seeligeri* and a *Listeria*-like (*Listeria* spp.) isolate, respectively (Table 3). These colonies were motile at 25°C and 37°C and were positive for both β -hemolysis and CAMP. One suspect isolate was classified as *L. seeligeri* because all three of the sugar reactions were negative. The xylose reaction was the only test that deviated from what the typical reaction is for *L. seeligeri*. The *Listeria*-like isolate was mannitol positive, +/- for rhamnose as well as for xylose meaning that either could be positive or negative, which may subsequently change the identity of the colonies. The two calf oral isolates were both isolated on MOX medium, both biochemically found to be *L. monocytogenes*, and both had umbrella motility at 25°C and 37°C (Table 3). However, these isolates ribotyped differently, which may indicate the close relatedness of *L. innocua* and *L. monocytogenes*.

Listeria RTs were not obtained from calf hair isolates analyzed. Two colonies phenotypically showed the presence of *L. monocytogenes* and *L. innocua*, but these ribotyped as *Staphylococcus sciuri* RT (4199) and *Enterococcus durans* RT (6210), respectively (Table 3). The suspect *L. monocytogenes* colony showed a typical biochemical reaction (Table 1), but typed as *Staphylococcus sciuri*. This same *Staphylococcus* RT was also found from a bedding isolate (Table 3). The bedding containing the *Staphylococcus sciuri* RT was not taken from the same calf pens in which the calf hair *Staphylococcus* RTs resided. The two hair RTs of *Staphylococcus sciuri* were not obtained from the same calf. This may indicate that if *Staphylococcus* or another organism such as *Listeria* is widely present in cow or calf bedding it may be transferred to their hair coat via contact when lying down in the bedding. The other hair isolate was phenotypically found to be *L. innocua* due to the negative sugar fermentations and the negative virulence tests,

which are the typical reactions for *L. innocua* (Table 3). Genotypically, calf anal isolates analyzed did not reveal the presence of *Listeria* RTs. One calf anal isolate suspected of being *L. seeligeri* ribotyped as *Staphylococcus sciuri* RT (4207) (Table 3).

River water

The topography of the farm and its proximity to the Tennessee River, as well as the direction of flow of the river, may be seen in Figure 1. Three sites were sampled: river water before the farm, which was referred to as the baseline because this first site was upstream from the farm. Thus, effluent from the farm did not play a role in contamination of samples taken from this site. The river water adjacent to the middle of the farm (0.7 km) and the river water below the farm (1.2 km) were second and the third sites selected for sampling, respectively. Ribotyped river water isolates from the baseline site were distinguished as *L. welshimeri* RT (1072) and *Enterococcus mundtii* RT (6235). The isolate typed as *L. welshimeri* was previously confirmed as *L. welshimeri* through conventional microbiological analysis (Table 2) due to its close relatedness, with the exception of the positive β -hemolysis reaction, to the typical reaction for *L. welshimeri* (Table 1). *Enterococcus mundtii* RT (6235) was phenotypically identified as a *Listeria*-like colony (*Listeria* spp.) due to its positive β -hemolysis, CAMP, catalase, and Gram reactions, as well as the presence of umbrella motility at both 25°C and 37°C. However, all three sugars demonstrated a positive fermentation reaction (Table 3). Two isolates from MOX medium of river water taken at the middle sampling site were conventionally distinguished as *L. monocytogenes* as a result of the positive CAMP test and +/- rhamnose test, which proved to be the most reliable of the sugar fermentation tests conducted in this research. These colonies typed as *L. welshimeri* RT (1074) and *L. welshimeri* RT (1079) (Table 2). Lastly, a river water isolate recovered at the end of the farm showed the presence of *L. monocytogenes* RT (1052), which was not found among any of the animal or farm samples subjected to ribotype analysis in this study.

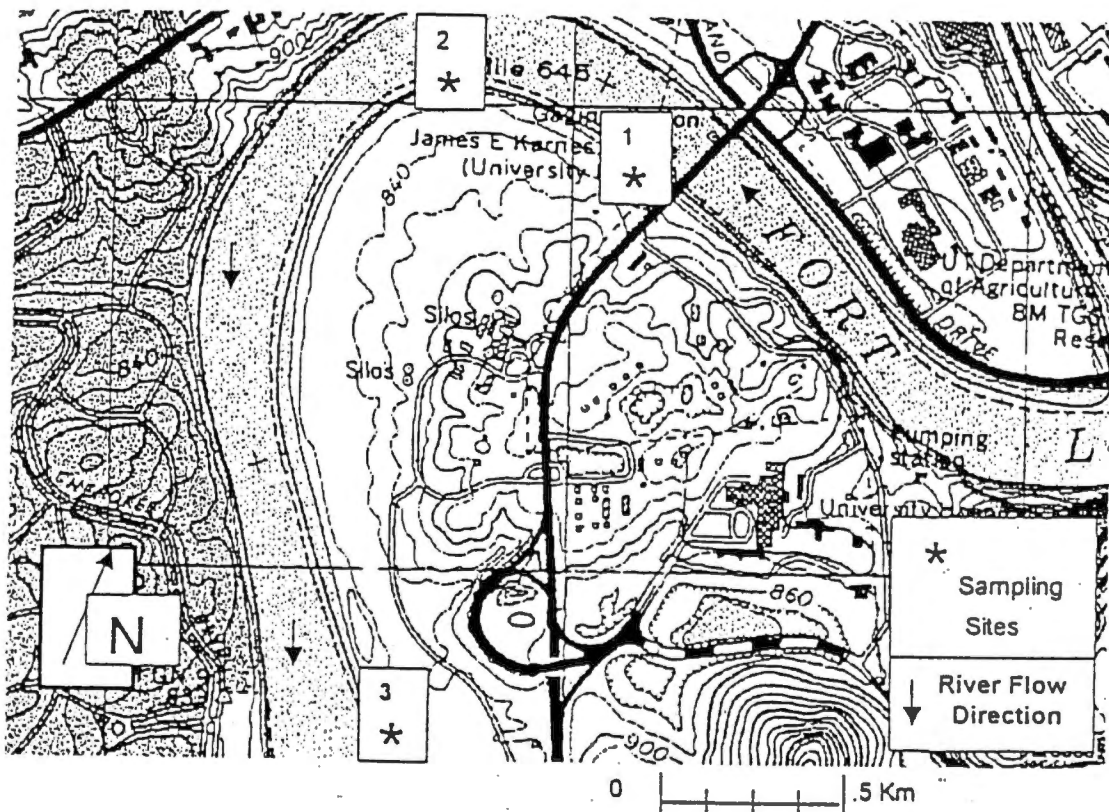


Figure 1. Topographic map of Tennessee river water sampling sites in relation to the dairy farm

The *L. monocytogenes* RT (1052) was phenotypically classified as a *Listeria*-like (*Listeria* spp.) colony because motility (indicated by colonies fanning out from the stab line in culture medium) and not classic umbrella motility was detected at both 25°C and 37°C. Additionally, the β -hemolysis and CAMP reactions were both positive (Table 2). Another colony from river water at the end of the farm was genotypically found to be *Bacteroides thetaiotaomicron* RT (12564). This sample was phenotypically negative for all fermentation and virulence (β -hemolysis and CAMP) tests, demonstrated motility at 25°C, and was not motile at 37°C (Table 3). These reactions are typical for *L. innocua* (Table 1) with the exception of the motility at 25°C and the fact that the Gram stain was negative. This isolate was still selected for ribotype analysis because of the Gram-positive rods seen among some of the Gram-negative rods in the light microscope field-of-view.

Feeds and Trough water

Analysis of two colonies from different feedbunk (a mixture of grain, hay, and silage) samples indicated the occurrence of *L. welshimeri* RT (1073) and *L. innocua* RT (1009) (Table 2). Two corn silage isolates obtained from the same sample revealed *L. monocytogenes* RT (1XXXX) (Table 2). The *L. monocytogenes* RT found in silage was not found among other farm source isolates ribotyped. These two corn silage isolates were confirmed as *L. monocytogenes*, although both colonies differed from the characteristic reaction for *L. monocytogenes* via umbrella motility at 37°C. One of these silage isolates fermented xylose (Table 2), which is unusual for *L. monocytogenes*. In a study by Ryser and colleagues (1997), *Listeria* spp., previously isolated from 13 of 129 corn silage samples were ribotyped. Three of the 13 were identified as three different distinct *L. monocytogenes* ribotypes (Ryser et al., 1997). The other 10 *Listeria* spp. isolates ribotyped as *L. innocua* (four different distinct RTs) (Ryser et al., 1997). Investigating the diversity of *Listeria* ribotypes recovered from dairy cattle, silage, and dairy

processing environments, Arimi and associates (1997) analyzed 44 silage isolates and found 6 *L. monocytogenes* RTs, 37 *L. innocua* RTs, and 1 *L. welshimeri* RT.

One RT from grain was found to be *L. innocua* RT (1006) (Table 2). This same RT (1006) obtained from a grain sample was also ribotyped from two different insect isolates (Table 2), verifying the casual relationship between insects and the potential for spreading contamination to various sites of the farm environment. Feed and silage may be contaminated by particles of feed that drop from animal's face and mouth back into the feeding trough where an environment exists that allows the proliferation of *Listeria* spp. These feeding areas can also be contaminated by the discharge of infectious bodily fluids from the animals' mouths, nares, or conjunctiva during the feeding process. In addition to contaminating feeds and silage, bodily secretions infected or colonized with *Listeria* spp. may also contaminate wooden feeding troughs.

The feedbunk colony, which ribotyped as *L. welshimeri* RT (1073), and the grain isolate, which ribotyped as *L. innocua* (1006), were both presumptively identified as *L. monocytogenes* (Table 2). They had typical fermentation patterns as *L. monocytogenes* (Table 1) except for they displayed umbrella motility at both 25°C and 37°C (Table 2). *L. innocua* RT (1009) also found in the feedbunk had a classic the presence of umbrella motility at 37°C (Table 2). Another feedbunk isolate typed as *Bacillus subtilis* RT (9500), while an additional grain colony, typed as *Bacillus fusiformis* RT (14809) (Table 3). These samples were both phenotypically verified as *L. seeligeri*. The grain sample differed from the typical via the umbrella motility at 37°C, while the feedbunk was atypical due to the positive mannitol.

Genotypic analysis of an isolate obtained from the watering trough of cows revealed the presence of *Bacillus licheniformis* RT (6016) (Table 3). This colony was biochemically classified as “*Listeria*-like” due to its strong positive CAMP, β -hemolysis, and catalase tests. In motility culture media, each at 25°C and 37°C this isolate displayed motility and classic umbrella motility,

respectively (Table 3). However, this Gram-positive isolate yielded fermentation patterns of positive mannitol, negative rhamnose, and variable xylose.

Raw Milk and Milking Equipment

Three isolates from raw bulk tank milk were ribotyped. Two revealed the presence of *L. innocua* RT (1019) and *L. innocua* RT (1005) (Table 2). Interestingly, these two RTs came from the same sample, which were isolated on two different media, modified Oxford agar (MOX) and PALCAM respectively, indicating that multiple strains may co-exist within the same sample. Arimi and associates (1997) ribotyped 14 raw bulk tank milk isolates and detected 5 *L. monocytogenes* RTs and 9 *L. innocua* RTs.

As seen in Table 2, *L. innocua* RT (1005) had umbrella motility at 25°C and 37°C, positive Gram and catalase reactions, +/- rhamnose, and negative mannitol, xylose, β -hemolysis, and CAMP test. *L. innocua* RT (1019) was phenotypically identified as *L. monocytogenes* (Table 2) because the fermentation patterns and virulence reactions matched the characteristics normally seen by *L. monocytogenes* (Table 1). The third bulk tank milk isolate was confirmed as *L. seeligeri* (Table 2), however, this isolate was mannitol positive, which deviates from the typical reaction for *L. seeligeri* (Table 1). After ribotyping, this isolate was most closely identified as *Enterococcus faecalis* (Table 3). Ribotypes of milking equipment inflations, cups, and hoses did not reveal the presence of *Listeria*. Of the two colonies ribotyped from the milking equipment cups, one showed the presence of *Enterococcus faecalis* RT (15103), and the other was *Enterococcus casseliflavus* RT (6203), which were conventionally confirmed as *L. monocytogenes* and *L. grayi*, respectively (Table 3). Both of these isolates were positive for mannitol fermentation, motile at 25°C, and CAMP positive. The biochemically confirmed *L. grayi* were negative for acid production from rhamnose, β -hemolysis, positive for fermenting xylose and demonstrated umbrella motility at 37°C. The presumptive *L. monocytogenes* had a +/- rhamnose test, a negative xylose reaction, was positive for β -hemolysis, and motile at 37°C (Table 3). An

isolate obtained from the inflations typed as *Enterococcus faecalis* RT (62112), which had the same characteristics of an isolate obtained from the bulk tank milk (Table 3). The information presented here indicates that *Enterococcus* spp. may be present from the milking device on teats of cows through to the bulk tank. Fedio and Jackson (1992) state that contamination of bulk milk may occur in one of two ways; directly as a result of udder infections or shedding of the organism via the udder. The second way would be indirect contamination during unsanitary milking practices if the organism were present on the udder surfaces, in feeds, feces, or other environmental sources such as the bedding (Fedio and Jackson, 1992).

Farmyard soil

Four soil colonies typed were classified via the nearest neighbor command as *Bacillus sphaericus* RT (11038), *Bacillus clausii* RT (12533), *Escherichia coli* RT (3009), and *Bacillus sphaericus* RT (6096 and 14766) (Table 3). These RTs were conventionally identified as *L. monocytogenes*, *Listeria*-like (*Listeria* spp.), *Listeria*-like (*Listeria* spp.), and *L. seeligeri*, respectively. The first soil colony ribotyped as *Bacillus sphaericus* was isolated on PALCAM agar and biochemically confirmed as *L. monocytogenes*. This reaction was typical for *L. monocytogenes* with the exception of a partial fermentation of xylose. The isolate that typed as *Bacillus clausii* was phenotypically *Listeria*-like (*Listeria* spp.) with umbrella motility at 25°C, motility at 37°C, positive catalase, and Gram reactions. Although, problems may arise during classification because both the virulence tests for this isolate were positive, while all of the sugar tests were negative (Table 3). Another soil colony was genotypically shown to be *Escherichia coli*. The Gram reaction was negative, thus eliminating this isolate as being *Listeria*. Two colonies distinguished as two different ribotypes of *Bacillus sphaericus* were conventionally confirmed as *L. seeligeri* and *Listeria* spp. The reactions were very similar to the first soil isolate, which phenotypically was identified as *L. monocytogenes*, but typed as *Bacillus sphaericus*.

However, the soil colony classified as *L. seeligeri* showed umbrella motility at 37°C but not at 25°C (Table 3).

Bedding

Two bedding samples conventionally indicated the presence of *L. monocytogenes*. The first sample was isolated on MOX medium and was genotypically identified as *Acinetobacter baumannii* RT (15146), while the second, isolated on PALCAM medium was ribotyped as *Staphylococcus sciuri* RT (4199) (Table 3). The bedding colony that typed as *Acinetobacter*, was Gram-negative, thus eliminating it as being *Listeria*. *Staphylococcus sciuri* (RT 4100) was biochemically shown to be *L. monocytogenes* (Table 3) due to its positive rhamnose, β -hemolysis, and CAMP tests, as well as displaying umbrella motility at 25°C and motility at 37°C. The problem with *S. sciuri* (RT 4100) arises with the mannitol test, which was positive, while for *L. monocytogenes* it is typically negative (Table 1).

Airborne

Among air isolates there were no *Listeria* RTs identified. Three air colonies were biochemically confirmed as *L. seeligeri*, *L. monocytogenes*, and *L. monocytogenes*. Using the nearest neighbors bacterial identification, the colonies were shown to be *Paenibacillus alginolyticus* RT (10138), *Lactobacillus ruminis* RT (13095), and *Bacillus laevolacticus* RT (13658), respectively (Table 3). The three air samples were motile at 25°C and 37°C, positive for the Gram reaction, catalase, β -hemolysis, and CAMP tests. The isolate phenotypically confirmed as *L. seeligeri* was negative for the three sugar fermentations (Table 3). The typical reaction for *L. seeligeri* is positive catalase, xylose, CAMP, and β -hemolysis, and negative mannitol and rhamnose (Table 1). Since only one sugar reaction was not similar to the typical *L. seeligeri* reaction and this isolate was motile at 25°C and 37°C it was classified as *L. seeligeri*. The other two colonies were conventionally distinguished as *L. monocytogenes* from the same isolation medium (MOX) and

had the same reactions, which differed from the usual reactions for *L. monocytogenes* via the +/- mannitol reaction and only showing a partial fermentation of rhamnose (Table 3).

Insects

Genotypically, *L. innocua* RT (1006) was obtained from two insect isolates, both were isolated on MOX medium and both of these colonies had typical reactions for *L. innocua* with the exception to a positive β -hemolysis test (Table 2). There are several points of significance about these two ribotypes, which include: both show umbrella motility in culture medium at 25°C and 37°C, and *L. innocua*, which is typically positive for fermentation of rhamnose with a few known negative strains, had a positive rhamnose test for both colonies. The same RT that was identified from these two insect samples was also ribotyped from one of the grain samples taken from a feed trough on the farm (Table 2). This may indicate that the insects may be becoming contaminated via the grain in the feed trough and may be transferring the *L. innocua* to other sites on the farm. Insects have long been known to be vectors of disease. Insects on the farm frequent numerous sites and are able to transport bacteria via epidermal structures on their bodies and excrete environmental bacteria in their feces (Domenichini et al., 1992). Thus, insects have a high potential for the transmission of *Listeria* spp. throughout the farm. As was concluded by Domenichini and associates (1992), insects are both a reservoir and a vector of *Listeria* spp. and have the capability to transmit *Listeria* spp. by their presence on domestic and wild animals as well as human and animal food sources.

Phenotypic and genotypic comparison of *Listeria* isolates

According to ribotype analysis, optimal detection sites for the presence of *L. monocytogenes* were from the cows' teats, the oral cavity of calves, corn silage, raw bulk tank milk, and from the river water adjacent to the midpoint of the farm. However, prior to ribotyping, colonies were phenotypically analyzed via catalase production, sugar fermentation patterns produced by mannitol, rhamnose, and xylose, motility at 25°C and 37°C, as well as β -hemolysis

and the CAMP (Christie Atkins Munch-Peterson) test in the presence of *Staphylococcus aureus*. The usual acid production reactions for *L. monocytogenes*, *L. innocua*, and *L. welshimeri* can be seen in Table 1. It is important to note that a few strains of *L. innocua* and *L. welshimeri* will not demonstrate acid production from rhamnose (Table 1). *L. monocytogenes* (75.0%) and *L. welshimeri* (75.0%) RTs were of the usual sugar fermentation patterns (Table 2), while 87.5% of *L. innocua* RTs showed typical biochemical results (Table 2).

The classical β -hemolysis and CAMP reactions for *L. monocytogenes* are a positive CAMP test in the presence of *S. aureus* and a positive β -hemolysis, however, a few strains may be negative in the later case. *L. innocua* and *L. welshimeri* are negative for both of these virulence tests. *L. monocytogenes* RTs revealed that 87.5% were positive for both the β -hemolysis and CAMP tests (Table 2). *L. monocytogenes* RT (1041) was phenotypically negative for β -hemolysis and positive for the CAMP test. *L. innocua* RTs showed that 87.5% were atypical for β -hemolysis and CAMP. Only *L. innocua* RT (1005) demonstrated the usual reactions for these virulence tests (Table 2). Of the *L. welshimeri* RTs, 25.0% yielded positive reactions for both β -hemolysis and CAMP reactions. One *L. welshimeri* ribotyped isolate produced a positive β -hemolysis and a negative CAMP test, while two others gave a negative β -hemolysis and a positive CAMP reaction (Table 2).

Most *L. monocytogenes* RTs (87.5%) demonstrated classic umbrella motility (UM) at 25°C utilizing the motility medium S culture medium (Difco) supplemented with 1% 2,3,5-triphenyltetrazoliumchloride (TTC) (Sigma Chemical Co., St. Louis, MO). However, (50.0%) of genotypically confirmed *L. monocytogenes* isolates demonstrated UM at 25°C, and motility at 37°C, while 37.5% demonstrated classic UM both at 25°C and at 37°C. One *L. monocytogenes* RT did not display UM at either 25°C or 37°C, but was motile at both temperatures. *L. innocua* RTs (100.0%) showed UM at both 25°C and 37°C (Table 2). According to Kathariou and associates (1995), *L. monocytogenes* and *L. innocua* differ significantly in motility and flagellin

production at 37°C. Classic *L. monocytogenes* strains are practically non-motile and produce little or no detectable flagellin at this temperature, while *L. innocua* strains are frequently motile and produce substantial amounts of flagellin at 37°C (Kathariou et al., 1995). Of the four *L. welshimeri* RTs identified in this research, all displayed UM at both 25°C and only motility at 37°C (Table 2).

Ribotyping results revealed the occurrence of numerous other organisms present on dairy cattle and in the dairy farm environment that were occasionally difficult to differentiate from *Listeria* spp., which are easily eliminated from consideration by using a wet mount (tumbling motility) and conventional biochemical testing. The most numerous of these organisms were the *Enterococcus* and *Leuconostoc*. Classic *Enterococcus* exists on plants and insects and in soils. In general, *Enterococci* found on insects and plants may be from fecal matter or the environment (Jay, 2000). *Leuconostoc* is common to the gastrointestinal tract, milk, and animal hides (Prescott, 1996). All *Listeria* spp. hydrolyze esculin. Numerous cocco-bacilli, *enterococci*, and group D *streptococci* also hydrolyze esculin and compete well with *Listeria* in selective enrichment as well as on MOX or PALCAM. Ten isolates resembling *L. monocytogenes*, biochemically and on isolation medium, three ribotyped as an *Enterococcus* spp., two were *Lactococcus* spp., two were *Staphylococcus* spp., two were *Bacillus* spp., and another was *Acinetobacter* spp. (Table 3). Due to the numerous, uncharacteristic biochemical reactions observed among a diverse population of *Listeria* in this type of research, it is important to gram-stain each isolated colony. In addition, viewing the motility of each colony via wet mount (FDA/BAM) (Hitchins, 1998) should be performed to augment classical or rapid microbiological biochemical reactions used to identify and differentiate *Listeria*, such as API.

The use of established rapid automated molecular techniques such as automated ribotyping as well as the validation of conventional microbiological methods are necessary for the optimal and reliable recovery of *L. monocytogenes* and other *Listeria* spp. in farm and

environmental data. Knowledge of the agricultural ecosystem, the taxonomy of *Listeria* strains, and careful attention to detailed isolation and confirmation protocols were essential to prevent misidentification of *Listeria* spp. when initiating and conducting on-farm pathogen-reduction programs.

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VITA

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